The Photo-oxidation of chlorophyll and related substances when adsorbed on solid surfaces.

Thesis presented by ALICE MARGARET McFARLANE, B.Sc.,
for the degree of Doctor of Philosophy.

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CONTENTS.

		Page
1.	Introduction	1
2.	Experimental Methods	28
3.	Experimental Results	54
4.	General Discussion and Conclusions	134.
5.	Summary	155.
6.	Bibliography	156.

INTRODUCTION.

INTRODUCTION

Since the investigations to be described in this thesis are concerned solely with the oxidation of pigment preparations brought about by the action of molecular oxygen, as distinct from other oxidising agents, the mechanism should more strictly be termed one of "auto-oxidation". (1) Essentially, such a mechanism is believed to consist of the addition of molecular oxygen to reactive atoms of the oxidisable material with the formation of highly unstable peroxides.

These oxygen addition reactions may be of two distinct types: (a) reversible, in which the products show considerable dissociation when either the original pressure or temperature is altered, and (b) irreversible, in which the reaction complex, although non-dissociable, is often so highly unstable as to give rise spontaneously to further products.

Included in the small list of experimentally known examples of the first group are a few complex organic molecules containing a co-ordinately bound metal atom. Thus, the respiratory functions of mammalian and invertebrate blood depend respectively on the two pigments haemoglobin and haemocyanin, both of which possess this property of binding oxygen in a reversible manner. The exact nature of the union between pigment and oxygen and the role played by the metal//

metal, which retains its divalent state throughout the oxidation, have never been satisfactorily elucidated. Measurements of magnetic susceptibility (2) however have indicated that the iron atom is definitely involved in the uptake of oxygen.

In 1927, Kunz and Krees (3), synthesised an iron complex of indigo blue which they claimed to be capable of undergoing similar reversible auto-oxidation. Furthermore, in this case, the primary oxygen addition seems to be very loose and has a tendency to become more firmly bound.

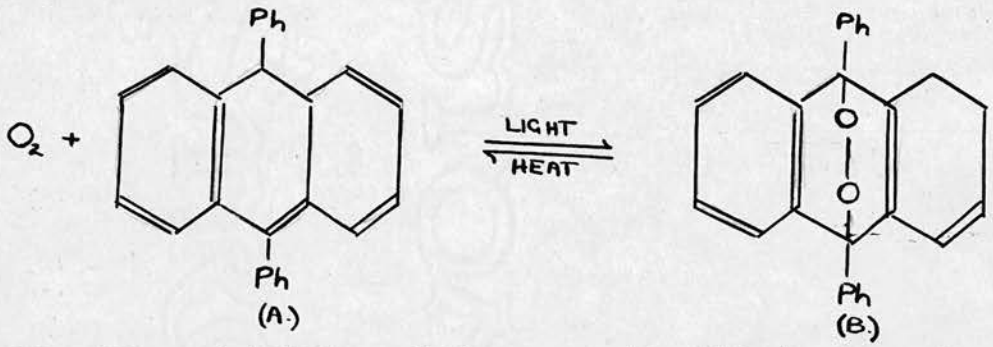
Milas (1) holds the view that in these complexes mentioned, the presence of the metal atom tends to activate neighbouring bonds and render them more susceptible to molecular oxygen.

More recently, several cobalt complexes have been reported as capable of undergoing reversible auto-oxidation, or "oxygenation" (4). Calvin and Diehl (5) are the principal workers in this field, the latter having investigated the possibility of using his material for the recovery of oxygen from the atmosphere. Another complex of the same metal was originally prepared by Werner, but its reversible properties were not discovered until 1948 by Michaelis (6).

Compounds entirely free from metals have also been known to show reversible auto-oxidation, the classical example of this group being rubrene, the red hydrocarbon synthesised by Moureau, Dufraisse and Dean (7). Of particular interest is the fact that this oxidation//

oxidation only proceeds at an appreciable rate when illuminated; it is followed by the evolution of heat, the bleaching of the hydrocarbon and the loss of its fluorescence. Dufraisse and his collaborators assert that there exists sufficient analogy between the reactions of haemoglobin and this hydrocarbon to claim that the oxygen is not, as previously assumed, bound to the iron atom of the pigment, but to some other unsaturated part of the molecule.

Later work showed that dissociating peroxides could be formed by numerous other polynuclear hydrocarbons containing the basic resonating system A, thus:-



(A.) (B.)

The dissociability of the peroxide (B) is dependent on the nature of the substituents in the meso-positions (8).

Consideration of the structure of the chlorophyll molecule, and the results obtained make it appear highly probable that chlorophyll itself belongs to this first group. Consequently, the second and larger group of non-reversible auto-oxidations will not be discussed at very great length. It is important, however, that they be mentioned, for this category//

category includes a great variety of organic compounds, many of commercial interest, from aldehydes and ethers to hydrocarbons, drying oils and rubbers (9). Oxidation to peroxides and their subsequent degradation products is believed in nearly all cases to follow a radical-actuated chain mechanism, promoted either thermally or photochemically (10). Metallic cations which are capable of undergoing a one-electron valency change are efficient catalysts for such auto-oxidations.

Almost without exception, the reaction schemes for such oxidations are exceedingly complex and vary with the nature of the substance being oxidised. Nevertheless, such variations can be considered as due to secondary reactions following a common initiation mechanism. Most of this work has been done in solution, which in itself introduces further complications.

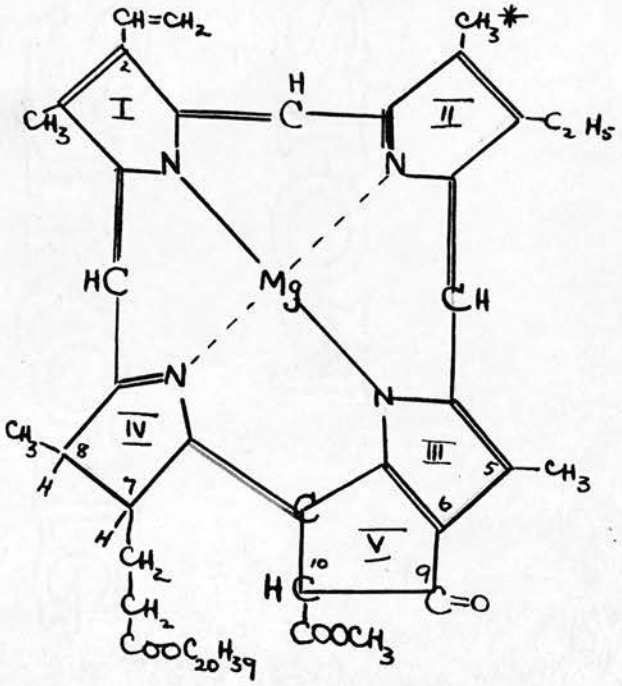
Chlorophyll.

The nature of the plant pigment has occupied the attention of scientific investigators for a great many years. The term "chlorophyll" was first used by Pellentier and Caventou who were also the first to realise the mixed identity of plant extracts.(11)

Largely owing to the exceptional instability of the chlorophyll molecule when exposed, even for short periods, to light, heat and the action of most chemical reagents, the prolific early researches yielded//

yielded little significant information.

Between the years 1906 - 1914, however, Willstatter and co-workers by devising reliable methods of extraction and purification, succeeded in clarifying many conflicting results (12). Eventually, by a method of differential solubility, they established in the green fraction obtained from plant extracts the existence of two components named chlorophylls a and b with the empirical formulae, $C_{55}H_{72}N_4O_5Mg$ for a and $C_{55}H_{70}N_4O_6Mg$ for b. Their work was confirmed and extended by Conant (13) and by Hans Fischer (14) until the latter's structural formula for chlorophyll a was almost universally accepted.



FORMULA I

Formula I is one of three isomeric (or mesomeric) variations of the structure proposed by Fischer, and is the form supported by Rabinowitch (15).

They//

They are distinguished only by the routing of the conjugated ring system and by the position of the semi-isolated double bond and the direction of the Mg - N bonds which depend on this routing.

In chlorophyll b, the methyl group indicated by an asterisk, is replaced by a formyl group.

As the formula indicates, chlorophyll is a magnesium derivative of dihydroporphin, esterfied at two carboxyl groups by phytol and methanol. Its relationship to haemin is thus quite obvious, since the latter is also a porphyrin derivative, the central metal in this case being iron.

It is not surprising that definite evidence as regards the structure of the chlorophyll molecule was not readily obtained, since the system appears exceedingly labile. Not only is the complexly bound magnesium atom easily removed by dilute acid solutions (16) but several groupings including the vinyl group at C 2, the two hydrogen atoms in positions C_7 and C_8, and the "lone" hydrogen atom at C_{10} are very susceptible to oxidation (17), while the presence of reactive double bonds offers opportunities for hydrogenations. Furthermore, according to Zscheile (18) certain changes in the molecule, undetectable by ordinary analysis become evident only by spectographic study.

Since 1930, innumerable papers have been published mainly by Conant and Fischer, but also by others such//

such as Rothemund and Knorr and Albers, (19) on the chemical reactions of chlorophyll. However, for present purposes, only those reactions relating to oxidations and photo-reactions of the pigment will be considered.

Most of the in vitro research has been carried out in solution; results therefore suffer from the disadvantage that the exact part played by the solvent is often difficult to estimate. This is especially true in the case of "allomerised" chlorophyll, formed when alcoholic solutions of the pigments are exposed to air. This has been interpreted as one of the simplest auto-oxidations of chlorophyll. The main difference between the unchanged and allomerised forms lies in their reaction, in the cold and in the presence of air, to alcoholic alkali. Ether solutions of intact chlorophyll give the "Molisch Phase Test", the transient apparition of a brownish colour, whereas allomerised samples under the same conditions do not show this colour change. Conant and coworkers concluded that one mole of oxygen is taken up in the slow allomerisation of one mole of chlorophyll in air (20). If the "brown phase" is indeed caused by an enolisation of the carbonyl group in position C9 involving the hydrogen from the neighbouring atom C10 (21), then it is reasonable to suppose that the non-appearance of this phase in allomerised chlorophyll is due to some binding of this "mobile" hydrogen//

hydrogen. Fischer (14a) therefore explains the uptake of one molecule of oxygen by assuming the formation, at C_{10}, of a chlorophyll peroxide:-

$$-\overset{|}{\underset{|}{C}}_{10}-H + O_2 \longrightarrow -\overset{|}{\underset{|}{C}}_{10}-O-OH \quad \ldots\ldots (1)$$

Although this peroxide has never been isolated, the auto-oxidation of this carbon atom is indicated by the isolation of a degradation product containing a hydroxyl group at C_{10}. However, since no allomerisation occurs in the absence of alcohol, Rabinowitch (22) considers it likely that reaction (1) is completed by the transfer of oxygen to alcohol, thus:-

$$-\overset{|}{\underset{|}{C}}_{10}-O-OH + CH_3OH \longrightarrow -C_{10}-OH + CH_3-OOH \quad \ldots\ldots\ldots (2)$$

It is still uncertain therefore, whether allomerised chlorophyll is a genuine peroxide or a "hydroxychlorophyll" as indicated by reaction 2. Whether or not this auto-oxidation is reversible is also uncertain, although Stoll and Wiedman asserted that allomerisation could be reversed, in the b -series at least, by mild reducing agents (23).

In 1937, Rabinowitch and Weiss (24) discovered that methanol solutions of chlorophyll were immediately bleached on the addition of ferric salts, the colour being restored by the addition of a ferrous salt, or other reducing agent. For various reasons, they attributed this effect to a reversible oxidation of the chlorophyll by the ferric ions:- $chl + Fe^{+++} \rightleftharpoons ochl + Fe^{++}$ where ochl represents//

represents the oxidised form of chlorophyll. This form is unstable, illumination with blue light or contact with water giving rapid irreversible bleaching.

The most readily observable and consequently the most exhaustively studied reaction of chlorophyll is its bleaching on exposure to light and air. As early as 1788, Senebier noted that leaf extracts were sensitive to light (25). Most authors have assumed that bleaching is caused by photo-oxidation, basing their assumption on results which showed firstly that chlorophyll solutions do not bleach in the absence of oxygen (26) and secondly, that there is an absorption of oxygen during bleaching (27). However, since these researches were carried out in solution, it is again not certain whether chlorophyll is indeed the final oxygen acceptor. According to Rabinowitch (28), bleaching is a change which is probably caused by a complex series of transformations, often involving the solvent or impurities, rather than by a single, well-defined chemical reaction.

It is appropriate to mention at this stage the experiments carried out by Livingstone and co-workers (29) on the reversible photobleaching of chlorophyll in methanol solutions, observed only in complete absence of oxygen. Again a solvent effect is in evidence, no measurable bleaching occuring in pure dry hydrocarbons in absence of polar molecules. When oxygen is present, the bleaching is completely irreversible. Rabinowitch has//

has suggested (30) that this reversible bleaching has properties in common with the bleaching of chlorophyll solutions in the presence of ferric ions and with the first stage of pheophytinisation.

Chlorophyll in vivo is more photostable than in vitro (31). Nevertheless, it can be bleached in the cells after prolonged illumination, provided oxygen is present. The reaction apparently only occurs on the exhaustion of the cellular reserve material. As for the chemistry and kinetics of the process, very little is known, the only work as yet reported having been carried out by Noack (32).

Of value, from the point of view of this thesis, are the few results which have been published on the bleaching of solid preparations of chlorophyll. Wager, working with thin films of chlorophyll adsorbed on paper or glass, found that oxygen was essential for bleaching and further, that a definite oxygen uptake took place. (33) His attempts to reverse the bleaching by the action of reducing agents were unsuccessful. The nature of the bleachd products, an important matter from the general view of photosynthesis, has aroused great controversy, but is still unknown. Usher and Priestley (34) alleged that hydrogen peroxide and formaldehyde are formed on illuminating chlorophyll, adsorbed on gelatine films, in the presence of carbon dioxide. This claim was later discredited by several workers, including//

including Warner (35) who proved that any formaldehyde formed on the illumination of chlorophyll films in air originates in the oxidation of chlorophyll and not in the reduction of carbon dioxide. However, the whole question of formaldehyde production was repudiated in 1918 by Willstatter and Stoll, who obtained negative results and questioned the purity of the preparations used by the earlier workers. (12)

Solid films of chlorophyll have thus been prepared and illuminated previously on glass, paper, gelatin and even chalk (36), but the respective investigators were concerned primarily, as indicated above, with the probability of the formation of organic compounds believed to be involved in photosynthesis, very little attention being directed to any quantitative estimation of the oxygen uptake or to the kinetics of the reaction.

In the present investigations, preliminary experiments with films of chlorophyll deposited on various solids as absorbents showed that photo-oxidation proceeded, but at exceedingly slow rates. Films deposited on a photoconductive substance, such as thallous bromide or thallous iodide however, showed quite promising rates of oxygen uptake, and it was therefore decided to continue investigations on the photo-oxidation of solid chlorophyll, especially when adsorbed on thallous bromide.

This substance resembles silver bromide in that it forms a latent image when illuminated by blue light in//

in gelatin suspension, the gelatin acting as a bromine atom acceptor.(37) In the absence of such an acceptor, no latent image appears to be formed, although it is possible theoretically that in vacuo, bromine vapour is liberated.(38) It was never detected however, under the conditions existing in the system employed.

A connection is apparent here with the photochemistry of other dyestuffs whose bleaching has been studied when absorbed on compounds such as titanium dioxide and zinc oxide, known to be semi-conductors, (39).

Chlorophyll as a photosensitiser.

The most important property of chlorophyll is its ability to act as a photosensitiser. It can perform this function not only in the natural process of photosynthesis, but also in innumerable in vitro systems (40).

Most of the investigations which have been reported are of a qualitative nature, and in some the photocatalytic character (41) of the reaction is by no means certain, attention being usually directed to alteration in the substrates rather than to the possibility of a change in the supposed catalyst. The only quantitative in vitro work of any significance was carried out by Gaffron on the auto-oxidation of allylthiourea (42), and by Ghosh and Sen Gupta on the//

the sensitised oxidation of phenylhydrazine by methyl red (43). From this and later work (44) it appears that chlorophyll can sensitise oxidation-reduction reactions with oxidising agents other than molecular oxygen. Although many tentative mechanisms (45) have been put forward to explain results obtained in this line of study, the present state of knowledge does not justify any broad generalisations.

Chlorophyll in the living cell can act as a sensitiser for numerous reactions, quite distinct from the photosynthetic process. Amongst these are the auto-oxidations of cellular reserve materials observed under the action of excess oxygen or high light intensity, or alternatively, under conditions which inhibit photosynthesis (46).

Noack found that cell chlorophyll could also sensitise the auto-oxidation of externally supplied substrates, such as benzidine (32) and even sensitised reductions have been observed with living chlorophyll (47). It has been suggested that all these results could be correlated by postulating one primary photochemical process for all reactions sensitised by chlorophyll in vivo. The different final results would then be due to secondary transformations of the same primary products (48).

The specific function of chlorophyll and other pigments in photosynthesis, the most important photosensitised reaction, is an exceedingly controversial subject//

subject which has supplied ample scope for theorists in the past, but is still without any definite solution. Engelmann was the first to discover (49) that only pigment-containing cells could evolve oxygen on illumination, and that, besides chlorophyll, other pigments could function in the assimilatory process. However, plants are known which contain chlorophyll yet cannot carry out photosynthesis (50). Thus it appears that the presence of pigments is a necessary but not a sufficient condition for photosynthesis.

Despite the claims of early workers, complete photosynthesis, meaning by that the reduction of carbon dioxide to the carbohydrate level and the oxidation of water according to the very general equation:- $CO_2 + H_2O \xrightarrow{h\nu} \{CH_2O\} + O_2$ has never been achieved outside the living cell. Such an equation however, gives only the overall results of photosynthesis, which is a complicated sequence of physical, chemical and photochemical processes, and not a direct reaction between carbon dioxide and water as indicated. Further, the reactants may not interact as such, but as part of larger "acceptor" molecules. Hill's works (51) on the oxygen evolution by chloroplast suspensions illuminated in presence of a ferric salt as oxidiser, showed that, in all probability the two fundamental processes in photosynthesis could be separated. His work was confirmed in 1942 by French and coworkers (52).

Such//

Such a conclusion is in keeping with the view of van Niel and Gaffron, who proved by their experiments with bacteria and anaerobically treated algae (53) that it is possible to substitute other reducing agents for water in photosynthesis. From such comparitive biochemical studies, these workers concluded that the two partial processes of oxygen evolution and CO_2 reduction are largely independent, and can be investigated separately. Ruben's work with isotopic oxygen (54) demonstrated clearly that all the oxygen evolved in photosynthesis came from the water molecules, none coming from carbon dioxide. This, correlated with the results of Wood and Werkman on carbon dioxide assimilation in non-pigmented systems in absence of light (55), makes it appear highly probable that the pigments have little to do directly with the uptake and transformation of carbon dioxide, but rather are concerned primarily with the liberation of oxygen.

Numerous reaction schemes based on the above deductions and on themeasurements of the quantum efficiency of photosynthesis have been published from time to time. These were summarised recently by Rabinowitch (56).

Of great interest is the actual mechanism by which chlorophyll participates in photosynthesis. It appears to have two alternative methods at its disposal:- (a) by the absorption of radiant energy and the physical transfer of this energy to the chemical//

chemical system, or (b) by actual chemical participation in the oxidation reduction reactions involved.

For various reasons (57), Rabinowitch believes it possible that both molecules which take part in the primary photochemical reactions sensitised by chlorophyll are permanently associated with the pigment in vivo. The existence of such a complex could explain the difference in fluorescence and photosensitisation results obtained in vivo and in vitro. Method (a) the physical transfer of energy to a colourless acceptor is less improbable when the reactants are bound in such a complex, but Rabinowitch believes nevertheless that the second alternative is still the more feasible. The earliest proponent of this latter scheme was Timiriasev who postulated that chlorophyll existed in two modifications which take part chemically in photosynthesis (58). Other theorists who supported this view tried to incorporate an interconversion of the a and b forms of chlorophyll into photosynthetic schemes (59). However no simple in vitro conversion has ever proved possible, and a further argument against this aspect of the theory is the fact that some photosynthesing algae contain no chlorophyll b (60). An alternative theory was proposed by Conant who suggested that chlorophyll was oxidised thermally by carbon dioxide in vivo to allomerised chlorophyll, which was then reduced photochemically by reaction with water. Stoll added the//

the modification that both reactions were photochemical (61).

The reversible oxidation of chlorophyll to a decolourised form, or the occurrence of chlorophyll in two interconvertible green forms belonging to two different levels of oxidation would, if definitely established, provide strong support for such theories. It was previously mentioned in this introduction that Rabinowitch (24) reported such a reversible oxide of chlorophyll, but its instability to water and light make its incorporation in photosynthesis improbable, unless conditions in the cell are such as to stabilise it in this form.

In summarising these arguments, it must be emphasised that no reversible photochemical interconversion has ever been demonstrated for any of the green forms of chlorophyll, and according to Livingstone (62), there is as yet no direct evidence to support the view that chlorophyll in acting as a photo-sensitiser is alternately oxidised and reduced.

A property of chlorophyll which has often been related to photosynthesis is its fluorescence. In intact plants, the fluorescence of chlorophyll is less than 1%, yet in solution the absolute yield is 10% (63). The effect of the solvent is however considerable, recent work by Livingstone attributing this to complex formation between the chlorophyll molecules and any hydrogen-bonding material present, (64). The//

The fluorescence of chlorophyll can be quenched by a variety of reagents, for example oxygen, methyl red and iodine, and in certain cases it has been suggested that a compound is formed between the dye and the quencher (65). Kautsky (66) claimed that his observations on the kinetics of chlorophyll fluorescence indicated the production of firstly, an unstable oxygen complex of chlorophyll, and on further illumination, a non-dissociable chlorophyll peroxide. However, since there is no departure in the case of oxygen from the Stern-Volmer law (65), such compound formation seems unlikely under these conditions.

The most recent hypothesis to explain why a rise in the fluorescent yield of chlorophyll is always connected with a limitation of the photosynthetic activity was advanced by Franck (67). Briefly, he believes that narcotic-acting substances, e.g. intermediate oxidation products made and destroyed by the oxidation processes, can be produced in plants and adsorbed at the chlorophyll-protein surfaces, thereby reducing the photochemical activity of the chlorophyll and enhancing the fluorescent yield. However, Livingstone (62) reports that no support for the belief that fluorescence and photosynthesis are complementary actions can be obtained from any in vitro experiments on chlorophyll.

The methods by which carbohydrates are built up after the initial photochemical reactions is a study in itself and does not bear directly on the present work.//

work. Progress towards the solution of this problem was achieved in recent years by the use of radioactive tracers. The first steps were taken by Ruben and Kamen (68) using C^{11}. Since then, two research schools have continued the work using the more stable isotope C^{14}. Of these, the Chicago group (69) have substantiated the conclusion reached in the earlier researches, that the chemical behaviour of the photosynthesised compound indicates a high degree of hydroxylation and carboxylation in a polyfunctional compound whose molecular weight is between 700 and 1500.

In remarkable disagreement with this, are the results reported by the Californian school, under Calvin; these results, obtained with $C^{14}O_2$, show that the first carbon dioxide acceptor in the photosynthetic process is very probably vinyl phosphate, since the first photosynthesised product observed was 2-phosphoglyceric acid (70)

It is impossible to decide in favour of one or other of the proposed schemes since the work is still in its early, partially completed, stages.

Physical Interpretation of Photochemical Reactions.

All photochemical reactions are composed of primary and secondary acts, these being distinguished as follows:- primary acts consist essentially in the absorption of the photon by the molecule concerned and any immediate results involving the molecule and its close environment, while secondary steps are the later chemical and physical processes which lead to the formation of the final chemical products. Most research in the chlorophyll field has been directed to the secondary acts, those complex sequences of reactions which transform the reactants, carbon dioxide and water, into the final plant products.

Knowledge which does exist however, of the spectroscopic and other photochemical properties of chlorophyll has yielded some information as to the primary steps in the molecule's reactions. (71) Such facts can be summarised most conveniently (72) by a conventional energy level diagram (Fig.1), or by a schematic potential energy diagram, as depicted in Fig.2. (73).

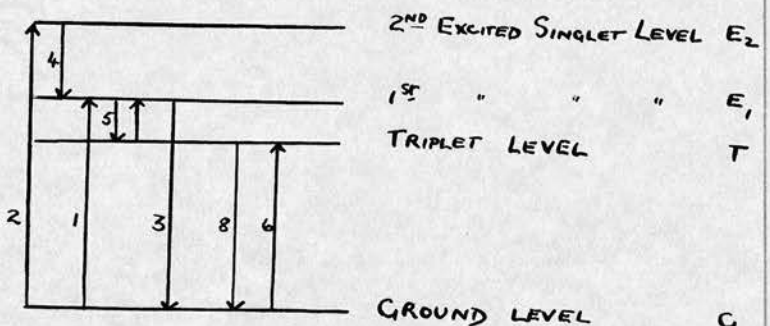


FIG. 1.

In Figure 1., only the electronic energy levels are depicted, and the possible transitions of the chlorophyll molecule are represented by arrows, as follows:-

Arrow 1: Transition from the ground state to the first singlet excited level. This occurs on the absorption of a quantum of red light. Chlorophyll in this state can lose its energy again by fluorescence (arrow 3) or by an "internal conversion" (74), followed by stepwise degradation.

Arrow 2:, Excitation of chlorophyll to the second singlet level on the absorption of a quantum of blue or violet light. Chlorophyll, like other pigments, does not exhibit fluorescence corresponding to this state; instead, some internal conversion occurs and via steps 4 and 3, red fluorescence is emitted, but with a low efficiency, the remainder of the energy appearing either as chemical energy or as heat, depending on the molecule's environment. Using the potential energy diagram (Fig 2.) a more accurate picture of the actual point of an internal conversion is obtained.(73.)

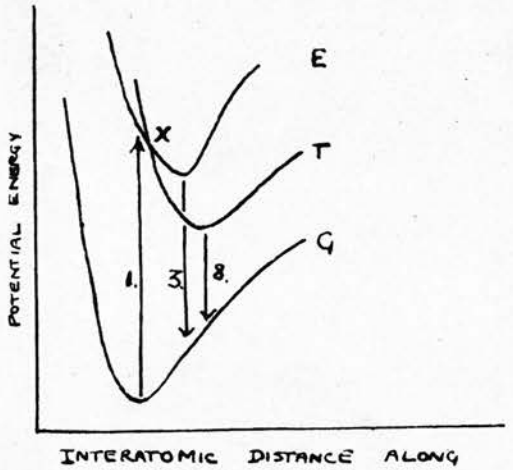


FIG. 2.

Here the three lowest electronic states of a complex molecule are represented by their potential hypersurface cross-sections along a critical co-ordinate. An internal conversion is only possible when the system is in a state such as at the point X, the intersection of two of these hypersurfaces.
Arrow 5: To explain the high yield of photosensitised reactions obtained by Gaffron (42) and others (44.), it is necessary (75.) to assume the existence of a long-lived state of the chlorophyll molecule, and that the majority of the excited molecules which are non-fluorescent, go via an internal conversion (arrow 5) to this state. The direct excitation to this level (T) from the ground state (step 6) is a "forbidden" transition (72) with a very low probability of occurrence. Lewis (76a) has reported that in certain complex molecules, this long-lived excited form is identical with a triplet state. His work on the phosphorescence of dyes in rigid solvents has shown that there are two processes by which the triplet state T may return to the normal state G:-
(a) By a reversal of step 5, brought about by thermal activation if the levels E, and T are sufficiently close, followed by step 3. This produces the emission of the "d-band", which is spectrally identical with normal fluorescence, although it has a relatively long mean life.
(b) By step 8, the only method possible at low temperatures//

temperatures. The molecules radiate after a long mean life, which is independent of temperature. The result is the emission of a band of longer wavelength and duration, termed by Lewis, "β -phosphorescence" (76b).

Because of its longer lifetime, the triplet state is believed to be the most photo-active of the molecule. Definite proof of its existence in the case of chlorophyll would therefore be of great value. Various attempts (77) have been made to detect the phosphorescence of chlorophyll, but the recent researches carried out in Livingstone's laboratory (78) have cast considerable doubt on the claims made by earlier workers.

As the excitation to a triplet state of a complex molecule requires the uncoupling of the spins of a pair of unsaturation electrons (73), such a state must correspond chemically to a diradical. The paramagnetism of organic molecules in this state can be taken as definite support for the theory (73).

The existence of such an energy-rich tautomer, or diradical, was suggested in 1941 by Franck and Livingstone (75) to explain various phenomena in a more satisfactory manner than was possible by the Kautsky theory of "metastable states" of dye molecules (77). The latter theory, which also postulated the transference of energy from the excited dye to the oxygen molecules present, gave rise to a great//

great many controversies (79). From several aspects, the tautomer theory is preferable to that of Kautsky, although Egerton (80) believes that an apparent similarity between the photochemical activity of dyes and white pigments, such as zinc oxide and titanium dioxide (39), argues in favour of the latter postulate.

Innumerable reaction schemes have been published, interpreting the transformations discussed above in terms of chemical equations. Perhaps the most acceptable is that put forward by Livingstone (81) to explain his reversible bleaching effect. It is also consistent with the features of fluorescence quenching and photosensitisation:-

1. $GH \xrightarrow{h\nu} GH^*$
2. $GH^* \longrightarrow GH + h\nu_f$
3. $GH^* \longrightarrow GH'$
4. $GH' \longrightarrow GH$
5. $GH + GH' \longrightarrow 2GH$
6. $Ox + GH' \longrightarrow G + OxH$
7. $OxH + G \longrightarrow GH + Ox.$
8. $O_2 + GH' \longrightarrow GHO_2$
9. $GHO_2 \longrightarrow GH + O_2$
10. $GH + GHO_2 \longrightarrow GH + GO_2H.$

GH^* = chlorophyll molecule

GH^* = electronically excited chlorophyll

GH' = long-lived excited chlorophyll(tautomer)

Ox = oxidising agent, or possible the solvent.

GHO_2 = unstable oxide of chlorophyll.

GO_2H = irreversibly oxidised and bleached product of chlorophyll

The use of such schemes will be developed further in the "Discussion" section.

In//

In conclusion, a brief introduction to the method employed in the actual investigations described in this thesis will be attempted.

As was mentioned earlier, all oxidations were in this case carried out in the solid phase, the chlorophyll being adsorbed on thallous bromide, a conductive substance which greatly increased the rates of reaction. Nevertheless, the rates of such photooxidations were still very slow, and the amount of oxygen absorbed comparatively small. Consequently, an exceedingly sensitive method for determining reaction rates and oxygen uptake was essential. These requirements were met adequately by a Bourdon gauge system, which will be described fully later. This device enabled the small pressure changes due to oxygen absorption to be measured accurately during illumination, and has many advantages over colorimetric methods based on estimating the extent of oxidation by the consequent bleaching of pigment or dyestuffs (39a).

Further, by confining attention to the solid phase, many of the complications which arise in liquid phase work are largely eliminated. Such complications are due in part to the nature of the solvent itself, especially if it capable of undergoing oxidation or reduction (22), and also to the fact that, in solution, the dissolved reactant molecules are more mobile, giving rise to the complex secondary reactions encountered//

encountered in the liquid phase oxidation of hydrocarbons, etc., (10).

It is hoped therefore that studies of the light-initiated oxidations of such solid films may offer some simplification of the general picture of auto-oxidations.

The scope of the present investigation.

Earlier work (32) had established that the photo-oxidation of chlorophyll by molecular oxygen proceeded at a measurable rate when the pigment was adsorbed on thallous bromide or thallous iodide films and that oxygen was taken up in the molecular ratio of 1:1 with the pigment. Furthermore, the reaction was proved to be reversible to a certain extent.

The work described in this thesis was designed mainly:-

(a) To confirm the essential features of the oxidation of the original chlorophyll preparation.

(b) To study the effect on the oxidation of various additional reagents, such as mineral and plant acids, and cobalt catalysts.

(c) To investigate further the relationship existing between the carotenoids and chlorophyll.

(d) To study the oxidation of other preparations of chlorophyll and to compare these with the original.

(e) To examine the effect of carbon dioxide and water vapour on the oxidation of all preparations from the general viewpoint of photosynthesis.

EXPERIMENTAL METHODS.

Apparatus//

EXPERIMENTAL METHODS.

APPARATUS.

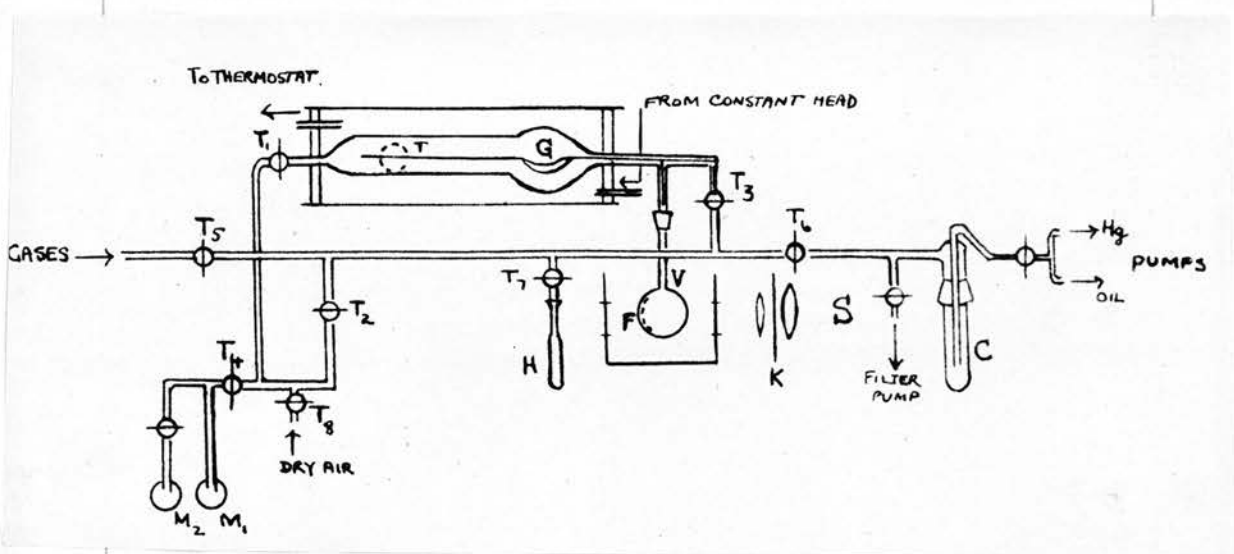


FIGURE I

The apparatus, shown diagrammatically in figure I, consisted essentially of a spherical reaction vessel V connected by a ground glass joint and capillary tubing to the Bourdon glass-spring gauge, G.

Oxygen and the other gases used in the experiments were stored in glass bulb reservoirs, and were admitted to the system through tap T_5. The pressure achieved was measured on the manometers M_1 and M_2, the latter being the standard. Water vapour, when required, was introduced directly to the vessel from the distilled water reservoir H, the pressure being balanced by admitting dry air through T_8 to the gauge jackets.

The whole system was constructed entirely of soda glass, all taps and ground joints being lubricated with Apiezon grease L, and it could be evacuated to a pressure//

a pressure of 10^{-3} m.m. of mercury by a CENCO HYVAC pump or to pressures of approximately 2.5×10^{-4} m.m. by a mercury diffusion pump, backed by the oil pump. In this connection, trap C was invariably surrounded by liquid oxygen, thus freezing out any pump oil or mercury vapour in the system. The residual pressures were measured on a McLeod gauge.

Source of light.

The source of light usually employed was an Osira mercury vapour lamp (125 watt) whose beam was focussed by a train of glass lenses on the pigment film deposited on the wall of the reaction vessel at F. Such a source gave a reasonably intense "white" light, no wavelength less than 3650 A penetrating to the system.

Calibration of the gauge.

The gauge was calibrated in terms of millimetres of mercury against M_1 and M_2 by observing what movement of the gauge pointer across the graduated eyepiece of the telescope T corresponded to a certain change in pressure on M_1. To obtain a measurable depression on M_1 several traverses of the pointer were brought about successively by altering the balancing air pressures of the gauge and its jacket.

For example, in one calibration, at approximately 200 m.m., 140 scale divisions were found to correspond to 4.4 m.m. measured on M_1. Similarly, at 100 m.m., 268 divisions corresponded to 8.4 m.m.

Therefore//

Therefore, in both cases, 1 scale divisions = 0.031 m.m. mercury, the accuracy of this measurement being ±.001 i.e. 3%.

The smallest pressure change which could be accurately observed was one tenth of a scale division i.e. 0.0031 m.m. mercury.

The sensitivity of the gauge in question was found to be independent of the actual pressure over the range 0-200 m.m. mercury.

Temperature Control.

Most of the experiments were carried out at 100 mm mercury pressure which, with such a sensitive pressure registering device, required the temperature controlling system to be as free from fluctuations as possible. To achieve this, water from a thermostat bath, regulated by a chloroform/mercury control, was pumped up to a large filter flask serving as a constant head, and from there led out in two streams, one flowing round the gauge jacket and the other round the reaction vessel. Their rates of flow could be regulated and were maintained at a constant value throughout any run. In both cases the water returned finally to the thermostat bath. All water-circulating parts were insulated either with felt or asbestos string, and the gauge jacket and vessel bath were well lagged with asbestos sheeting. With an adequate flow, the temperature fluctuation did not exceed $\pm 0.01^{\circ}C$ at any point.

Since//

Since it was impracticable to keep this thermostatic flow running overnight, a small mercury control was constructed. This, when used with an electric heating mat placed between asbestos boards under the vessel bath, maintained the latter's temperature at 25°C ±0.40°C. This was used only in certain experiments, for example, those involving the use of water vapour, where it was desirable to maintain the reaction vessel at approximately the correct temperature overnight.

Purification of the gases.

All the gases used were obtained from cylinders and in each case the gas was first dried by passing slowly over phosphorus pentoxide contained in a drying tube attached to the apparatus. In the case of oxygen, before the gas was allowed to enter the gas holder, any condensable impurities present were frozen out by passing through a trap surrounded by a carbon dioxide/acetone mixture.

Carbon dioxide, however, was admitted directly from the drier to the holder. It was then purified be freezing into a liquid oxygen trap, and pumping off any residual uncondensed gases. The purified carbon dioxide was then expanded back into the holder by removing the refrigerant, the process being repeated to remove any impurities trapped by the first freezing.

The//

The hydrogen used in these experiments was prepared in the laboratory by W.I.H.Winning, Ph.D.(83). The method involved electrolysing a 10% caustic soda solution containing a little baryta, the platinum cathode being isolated by a cylindrical glass tube. The gas was immediately purified by being passed slowly over copper gauze and platinised asbestos, both of which were electrically heated; it was then dried over phosphorus pentoxide. The final stages included passage over a tungsten filament at dull red heat, passage through a liquid oxygen trap and drying once again over phosphorus pentoxide.

Volume of reaction vessels.

The pressure changes obtained in any experiment refer to the volume of the isolated system consisting of the vessel, gauge and connecting tubing to tap T_3. The only means of determining this total volume was by expanding a mass of gas occupying it at a known pressure and constant temperature into a subsidiary vessel of known volume attached temporarily to the apparatus, measuring the pressure then obtaining, and applying Boyles Law. For convenience, the actual results obtained will be presented with the procedure.

Let V_1 = volume of reaction vessel + gauge.

Let V_2 = " " connecting tubing between T_3 and T_9.

Let V_3 = " " subsidiary vessel to tap T_9.

(see Fig. 2)

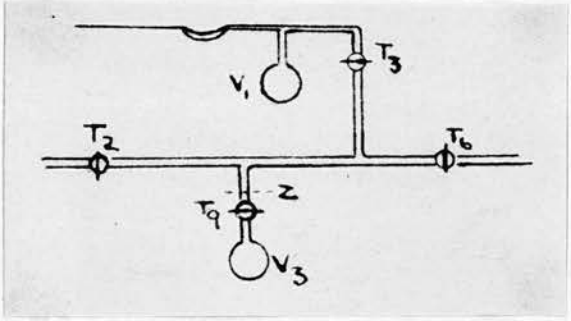


Figure 2.

Determination of V_3.

The subsidiary vessel, cut off at the point Z, was weighed empty, and then full of distilled water up to and including the capillary of tap T_9.

Thus:-

Weight of bulb V_3 empty	=	45.32 grams.
Weight of bulb V_3 + water	=	132.22 grams
Weight of water	=	86.90 grams.
Temperature of water	=	12.0°C
Volume of water	=	87.0 ml.

Detailed procedure.

(1). The apparatus ($V_1V_2V_3$ and gauge jacket) was filled with dry air at room temperature to a pressure of 544.8 m.m. = P_1.

(2) V_2 and V_3 were evacuated with T_3 closed.

(3) The gas in V_1 was then expanded to fill V_2, Tap T_9 being closed. The balancing pressure in the gauge jacket was reduced to keep the pointer in its initial position on the telescope scale. The new pressure, read on M_2 = 458.3 m.m. = P_2.

(4) With T_3 and T_9 open, the procedure was repeated, expanding//

expanding the gas in the volume V_1V_2 into V_3.

Final pressure = 249.0 m.m. = P_3.

Calculation.

By Boyle's Law, $(V_1+V_2+87.0)\ 249.0 = (V_1+V_2)\ 458.3$

$$\text{i.e. } \frac{V_1+V_2+87.0}{V_1+V_2} = \frac{458.3}{249.0}.$$

$$\text{i.e. } 1+\frac{87.0}{V_1+V_2} = 1.842$$

$$\text{i.e. } \frac{87.0}{V_1+V_2} = 0.842.$$

$$\text{i.e. } V_1+V_2 = \frac{87.0}{0.842} =$$

103.3 ml.

Also,

$$V_3 \times 544.8 = (V_1+V_2)\ 458.3$$

$$\text{i.e. } V_1 = \frac{103.3 \times 458.3}{544.8} = 86.9 \text{ ml.}$$

$$\therefore V_2 = 103.3 - 86.9 = 16.4 \text{ ml.}$$

Repetition of the experiment gave:-

V_2 = 16.35 ml. (average)

Volume of 1st reaction vessel + gauge = 86.9 ml.

" " 2nd " " " = 86.2 ml.

Choice of vessel size.

The volume of approximately 80 ml. for both the reaction vessels was not selected at random, but in an attempt to compromise between the conflicting requirements:-

(a) for a small vessel which would produce correspondingly//

correspondingly large pressure changes in any reaction, thus increasing the accuracy in measuring reaction rates.

(b) for a vessel of such size that the temperature controlling system used would still be adequate at 100 m.m. gas pressure, smaller vessels requiring rigidly exact thermostatic control. Any fluctuation in water temperature will show equally in the divided stream system, but the effects only cancel out if the gauge jacket and vessel volumes are similar.

Preparation of thallous bromide.

Solutions used:-

Thallous nitrate (analar) ..... 5.320 grams in 100 ml. water.

Potassium bromide (analar) ..... 5.000 -ditto-

50 ml. of thallous nitrate solution were added slowly from a burette to 25 ml. of the potassium bromide solution, with continuous shaking in dim yellow light. The quantities were chosen so that precipitation always occurred in the presence of excess bromine ions, the potassium bromide being 5% in excess of the calculated equimolecular amount. The precipitate was allowed to settle before being washed several times by decantation with distilled water. Is was then dried over phosphorus pentoxide, powdered and stored in a desiccator for several days before being used.

Preparation of chlorophyll Solutions. //

Preparation of Chlorophyll Solutions.

In all, three preparations of chlorophyll were used, two from pastes, composed of a mixture of plant pigments supplied by J.F. Macfarlan & Co., and the third obtained by direct extraction from spinach leaves. The first of the commercial pastes was found to contain copper, added as a stabiliser and present to the extent of 0.7%.

For clarity, these preparations will be designated as follows:-

Preparation: 1. Macfarlan's Copper containing preparation.
2. " Copper-free -ditto
3. Spinach chlorophyll.

Solutions were prepared from the commercial pastes by shaking approximately one gram of crude paste with 100 ml. acetone for a few hours in the dark, filtering off the insoluble residue which remained, and determining the concentration of the resulting stock solution.

After suitable dilution to give concentrations of approximately one gram per litre of acetone, the solutions of mixed pigments were used in certain experiments where it was thought desirable to have present both the chlorophyll and carotenoid fractions.

Chromatographic Purification.

Since the common adsorbents such as alumina have been known to cause degradation of chlorophyll (84), bonemeal was chosen, partly because of its relative inertness//

inertness in this respect and also because of its efficiency in separating carotenoids from chlorophylls (85.)

The adsorbent itself was prepared from crude steamed bonemeal by repeated extraction of impurities with hot alcohol, followed by exhaustive washing. The material was then dried and ground until it passed a 100 mesh sieve.

The column was prepared by the gradual addition of a slurry of the meal in petroleum ether (60° - 80°C), observing the usual precautions (86). The final dimensions were approximately 9 cm. x 2 cm. diameter. A glass wool plug prevented the adsorbent reaching the receiving flask, but the precaution of filtering the fractions before determining their concentration was always observed.

Before adding the chlorophyll solution, the column was well washed with petroleum ether of the same boiling point range.

20 mls. of the stock solution were evaporated to remove acetone, dissolved in the minimum of petroleum ether, and added to the column in a very dim light.

On developing with more petroleum ether, several regions or bands could be distinguished:-

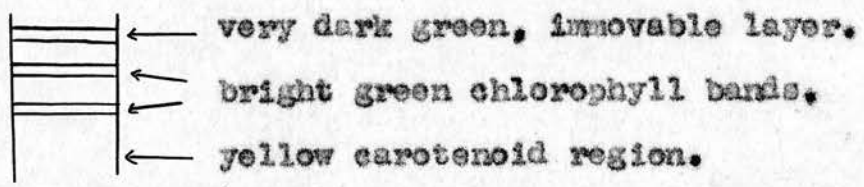


Figure 3

After//

After continued washing with approximately 300 ml. of the developing solution, the yellow fraction was completely removed, the eluate becoming colourless. The chlorophyll fraction was then eluted by adding acetone to the column, all but the topmost band washing out easily. Extensive washing at room temperature with several solvents, including ether, benzene, alcohol and acetone could not move this top layer, nor could any significant amount of green colour be extracted from it when it was extruded and boiled with acetone. It was therefore discarded, being probably a degradation product adsorbed very strongly by the bonemeal.

Comparison of the absorption spectra, as determined visually with Hilger Spectrophotometer, of the original stock solution and the purified green fraction, was used to confirm the removal of carotenoids. If necessary, the adsorption and washing were then repeated.

Extraction of chlorophyll from Spinach leaves.

The method used was a modification of that described by Zcheile (18) for the preparation of spectroscopically pure chlorophyll. One kilogram of fresh spinach was extracted with acetone, in batches of 250 grams to approximately 400 ml. acetone. A little calcium carbonate was added to each batch to neutralise any plant acids liberated. The extraction was achieved by vigorous shaking for several hours in dark bottles. The leaves were then filtered off on a large buchner funnel, //

funnel, washed with a little acetone, and the combined residues re-extracted until almost colourless. It was found necessary to reduce the volume of the extract somewhat at this stage, by pumping off acetone vapour at the filter pump. The concentrated solution was then treated with petroleum ether (B.P. 38-40°C) as follows:-

Approximately 30 ml. of the extract were added to 300 ml. petroleum ether in a large separating funnel, and the mixture shaken to distribute the chlorophyll equally between the two solvents. Distilled water was then added to produce two distinct layers. The flask was rotated gently and the lower water/Acetone layer removed. In this way most of the acetone and water-soluble impurities were removed. The volume was again reduced by evaporation of solvent at the filter pump. The petroleum ether solution was then scrubbed four times through distilled water to complete the removal of acetone. This was achieved by allowing the extract to run slowly from one litre separating funnel into another supported vertically below it, containing the distilled water. To ensure the slow steady flow necessary to obtain efficient washing, the bore of the first funnel had to be reduced to that of a 10 ml. pipette by attaching a piece of drawn-out glass tubing at A. (Figure 4). The tip of this jet was immersed 5-10 centimetres under the surface of the water in the lower flask.

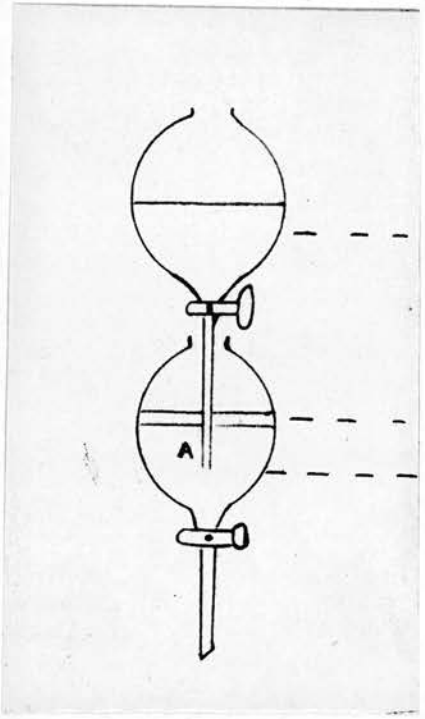


Figure 4.

The water layers were discarded and the petroleum ether solution dried over anhydrous sodium sulphate overnight. A sample of the resulting solution, containing crude chlorophyll, was filtered and adsorbed on a bonemeal column (14 cm. x 2 cm.) - prepared as described previously. The adsorbed chlorophyll was washed exhaustively with petroleum ether (60° - 80°) until the washings were colourless. This had removed most of the carotenoids, except one orange band (most probably xanthophyll) which still persisted at this stage (87). The column was then washed with a mixture of 60° - 80° petroleum ether/ ether in the ratio of 10:1 by volume. This caused the second green band and the orange band to move slightly downwards. Eventually, however the orange band separated from the green and was eluted.

This//

This was followed by a pale yellow-green eluate and the washing was therefore continued with the petroleum ether/ether mixture until the eluate was again colourless. The chlorophyll fraction, containing the a and b components, was then washed out with analar acetone and filtered to remove any adsorbent. Again, a green immovable band remained strongly adsorbed at the top of the column.

The absorption spectra of the acetone solution of chlorophyll was examined to confirm the removal of carotenoids, and to test the intactness of the preparation by comparing it with the results reported by Zscheile (18). Its fluorescence in ultra-violet light and its reaction, in ether solution, to cold alcoholic alkali, the "phase test", (88) were also examined.

Sugar chromatography.

Since Zscheile had recommended the use of sucrose in purifying chlorophyll, most adsorbents as already mentioned having a tendency to cause degradation, it was decided to attempt this method to see if the product differed in any obvious way from that obtained from the bonemeal column. 10 mls. of the crude chlorophyll solution in 38° - 40° petroleum ether were therefore adsorbed on a column, 25 cm. by 2 cm., prepared from a slurry of icing sugar in the same solvent. The sugar had been dried previously at 80-90°C in vacuum. The column was washed with petroleum//

petroleum ether until free from carotenoids.

The remaining chlorophyll, when eluted with ether and dried over anhydrous sodium sulphate, had an absorption spectrum which did not differ significantly from the bonemeal-purified sample.

All operations in the extraction including the chromatographic separation, were carried out in very dim yellow light. The solutions finally obtained were stored in the dark, and were used as soon as possible after preparation.

Determination of the concentration of pigment solutions.

This was achieved by evaporating, under vacuum, measured portions of the solutions concerned. The evaporation was continued for several days in a dark room, until constant weight was attained. A typical set of results was found to be as follows:-

Volume of stock solution taken = 20.0 mls.

Weight of crystallising dish empty = 19.7117 grams

Days.	Weight of dish.	Weight of chlorophyll residue.
3	19.8247	0.1130 grams.
6	19.8244	0.1127 "
10	19.8244	0.1127 "

The determination was invariably repeated with a fresh portion of the solution. When larger residues were involved, the solvent was removed at the oil pump, using a liquid oxygen trap. In this case also, evacuation was continued for several days.

Determination//

Determination of the copper content of the first chlorophyll sample.

(a) Copper content of crude paste.

Approximately 1 gram of the solid preparation was dried over phosphorus pentoxide, weighed and ignited in a crucible to constant weight. The ash was dissolved with gentle heating in approximately 1:1 nitric acid and diluted to 50 mls. in a standard flask. This solution was then diluted ten times to give a concentration of approximately 0.01 mg. copper/ml. Portions of the diluted solution were compared colorimetrically with a standard copper sulphate solution by the diethyldithiocarbamate method (39).

(b) Copper content of bonemeal purified chlorophyll.

30 mls. of the stock solution were evaporated and chromatogrammed in the usual way. The chlorophyll fraction obtained was evaporated, weighed and then ignited to constant weight. The ash was dissolved in a few drops of 1:1 HNO_3 and the solution diluted to 50 mls. with distilled water. The copper content of this solution was determined colorimetrically using the same method as above.

Determination of the absorption spectra of chlorophyll solutions.

Apparatus.

The Hilger Constant Deviation Wavelength Spectrometer in conjunction with a Barfit Hutting Photometer was//

was used for all absorption measurements.

The method adopted followed closely the general instructions listed by Twyman and Alsopp(90).

Since it was not intended to determine the finer details of the absorption curve, this having been satisfactorily accomplished already by workers such as Zscheile, Rabinowitch etc., but rather to observe any distinctions among the three samples used, and between the oxidised product and the original, readings were taken at 100 Å intervals over most of the range 7000 Å - 4600 Å, and at intervals of 50 Å in the region of the spectral peaks. The general principle governing the selection of cell length and solution concentration is that the regions of greatest interest, usually the absorption maxima, should be measured in the most accurate part of the density scale i.e. where $\Delta^{d}/_{d}$ is least.

With cells of exactly 1 cm. length, this was invariably achieved by using solutions of concentration 1.0 gram per litre diluted four to six times.

Calibration of the Instrument.

The wave-length drum of the spectrometer was calibrated by replacing the pointolite source used in the determinations, by an Osira mercury vapour lamp and adjusting the position of the wave-length pointer in the field of view, until it centred on the band of known wavelength selected by the drum pointer.

Preparation of Films.

(a) Choice of thallous bromide.

In preliminary experiments, various solids were used as adsorbing films for chlorophyll, but the rates of oxidation were so low that it was decided to try a strongly photo-conductive solid, namely, thallous bromide. The rates obtained were some ten times faster than with the "inert" substances, and consequently it was decided to use thallous bromide in further investigations.

The solids tried initially included zinc oxide, alumina, thallous phosphate, ferric phosphate, powdered Jena glass and talc.

(b) Variation of rate with amount of thallous bromide.

In varying the amount of thallous bromide used to prepare the film from 0 to 0.82 grams, it was found that a maximum initial rate was reached with 0.3 grams, the rate decreasing again with increasing amount of solid, this retardation being ascribed to screening or to increased scattering of light by the crystals. 0.3 gram of thallous bromide was therefore used consistently in all the experiments.

(c) Method.

0.3 gram of thallous bromide in a dry, finely powdered state, was carefully brushed into the dry reaction vessel. The required amount of pigment solution, usually 3.35 or 3.32 mls. of a 0.1% solution//

solution in acetone, was added from a calibrated pipette, whose narrow stem could project down the neck of the vessel, allowing the solution to drop directly on the powder. Most of the acetone was then removed by evaporation at room temperature at the filter pump, the flask being supported with its neck almost horizontal. Care had to be taken to avoid rapid boiling and subsequent spurting of the pigment over the glass of the vessel at these low pressures. When the solution had been concentrated to approximately 0.1 ml., the flask was detached from the pump and swirled carefully in such a way as to spread out the thallous bromide, giving a smooth film of approximately one inch in diameter on one side of the vessel. It was then re-attached to the pump and the evaporation completed while continuing the swirling motion to prevent the thallous bromide gravitating to the centre of the part chosen for the film. Even the most careful manipulation could not prevent some chlorophyll spreading over the thallous bromide and depositing on the glass. This could be transferred back to the film however, by successive rinsing with very small drops of acetone from the pipette, and evaporating as before.

If the oxidation of the chlorophyll or the carotenoids was to be studied in the presence of other reagents, e.g. phosphoric acid, the requisite amount of such a solution was added at this stage. When the reagent//

reagent in question was dissolved in a volatile solvent, this was removed at the filter pump, observing all the precautions detailed above. In the case of substances in aqueous solution, the water was removed at the oil pump, over a trap of phosphorus pentoxide.

The vessel was then attached to the apparatus, as shown in figure I, in such a way that the film would be completely illuminated. The system was evacuated for several hours, either by the oil pump or by the mercury diffusion pump. After standing evacuated overnight, tap T_4 being closed to prevent anypossibility of mercury vapour diffusing on to the film, it was subjected to a further evacuation of approximately one hour's duration before admitting oxygen and commencing a "run".

Procedure followed during a typical oxidation.

After the gauge and vessel system had been sufficiently evacuated, the glass leads connecting T_5 to the gas reservoirs were pumped out before oxygen was admitted to the apparatus. Initially, only a few millimetres were introduced and almost immediately pumped out again, to give the effect of "washing out" the system and removing gaseous impurities, for example, any persistent traces of solvent vapour. 100 m.m. of oxygen, as measured on M_1, were then slowly admitted. Care was required at this stage as the gauge was very liable to fracture, the//

the maximum tolerance being a difference in pressure between it and its jacket of approximately 4 millimetres of mercury. Although the gas was admitted equally to both sides of the gauge, the difference in path lengths caused considerable fluctuation of the pointer.

Taps T_1 T_2 T_3 were now closed, isolating the vessel and gauge from the gauge jacket. As soon as a constant temperature had been obtained in the thermostatically controlled parts of the apparatus, and the gauge pointer had reached a steady position, the shutter K was raised and illumination begun.

The position of the pointer was read on the telescope scale at intervals of ten minutes throughout the experiment and the results were graphed. From the pressure/time curve obtained, the rates of reaction at various values of oxygen uptake were calculated.

By subjecting the film to a continuous illumination and analysing the results in this way, the explanation for various complications which arose in the preliminary work became evident. When oxygen uptake was plotted against time, the resulting curve resembled that of auto-oxidations in general (91), consisting of an induction period, followed by a constant rate portion, this being succeeded finally by an asymptotic increase to a maximum representing complete oxidation.

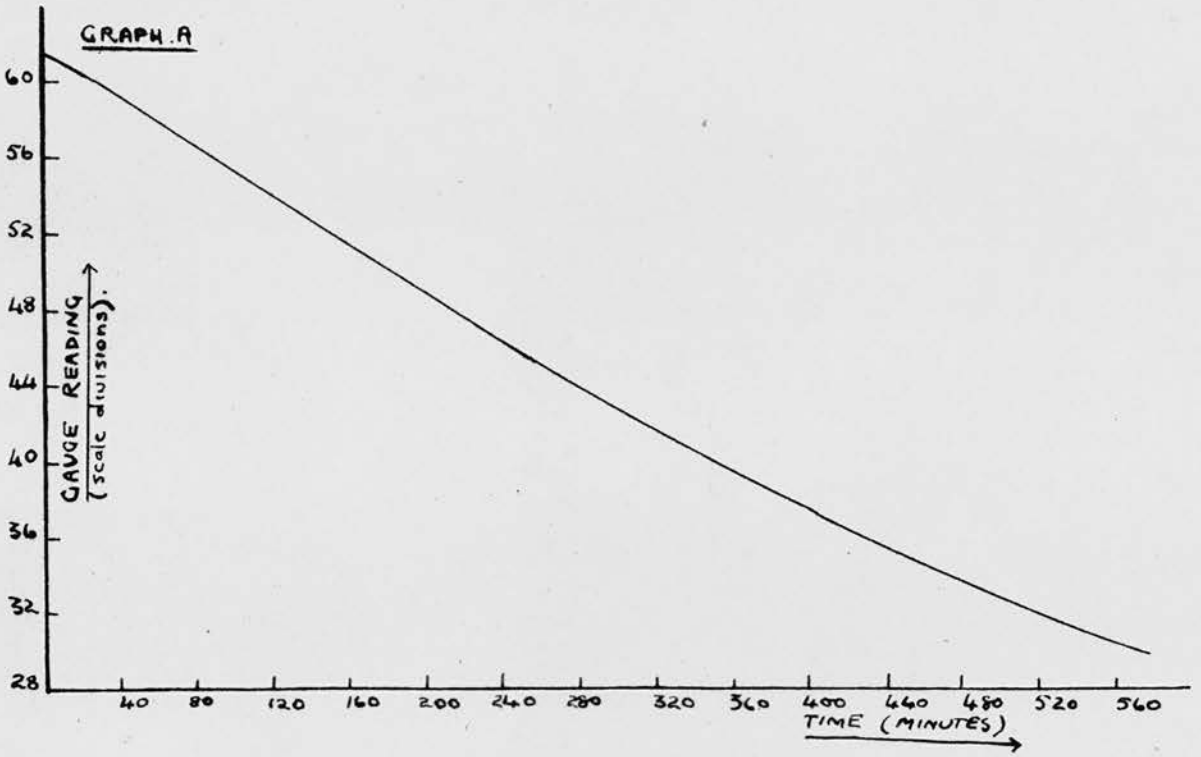
It was more convenient, however, to graph reaction rate against oxygen uptake, since, as will be explained//

explained later, this method gave a direct measure of the total oxygen absorbed in the oxidation. This graph will be generally referred to as the "Reaction Curve".

Since in any one experiment, no more than one or two millimetres of oxygen were taken up when the pigment present was fully oxidised, the pressure could be taken as constant at 100 m.m. The following set of readings, actually taken during one experiment, serve to illustrate the method:-

TABLE A.

TIME (MINS.)	GAUGE READING (SCALE DIVS.)	TIME	GAUGE READING	TIME	GAUGE READING.
0	61.3	181	49.9	404	37.6
8	61.0	190	49.5	411	37.1
13	60.9	202	48.7	416	36.9
23	60.3	215	47.7	421	36.7
32	59.9	225	47.3	437	35.9
44	59.0	228	47.0	446	35.3
54	58.4	238	46.5	453	35.0
64	57.7	246	46.1	462	34.6
69	57.2	257	45.4	476	34.0
80	56.7	273	44.5	489	33.6
89	56.1	289	43.4	505	32.9
97	55.7	312	42.2	517	32.3
102	55.3	324	41.5	525	32.0
111	54.7	328	41.2	532	31.9
122	53.9	337	40.9	547	31.1
141	52.7	345	40.3	559	30.8
152	51.9	359	39.6	564	30.6
156	51.7	367	39.2	573	30.2
161	51.3	373	39.0		
169	50.8	385	38.6		


GRAPH A
60
56
52
48
44
40
36
32
28
GAUGE READING (scale divisions).
40
80
120
160
200
240
280
320
360
400
440
480
520
560
TIME (MINUTES)

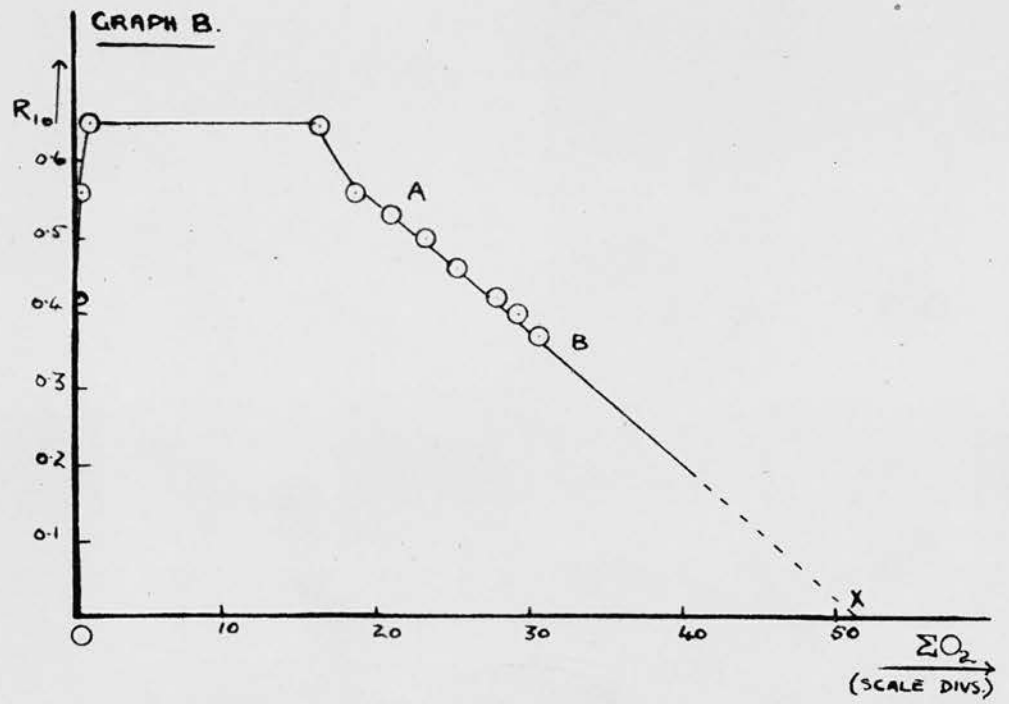

GRAPH B.
R_{10}
0·6
0·5
0·4
0·3
0·2
0·1
A
B
X
10
20
30
40
50
ΣO_2
(SCALE DIVS.)

TABLE B.

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.4	0.42	16.3	0.65	25.1	0.46
0.6	0.56	18.6	0.56	27.7	0.42
1.2	0.65	20.7	0.53	29.1	0.40
4.0	0.65	23.1	0.50	30.6	0.37
10.0	0.65				

Where ΣO_2 = oxygen uptake at any instant, expressed in scale divisions.

R_{10} = rate of oxidation at the same instant, expressed in scale divisions per 10 minutes.

Graph B then, represents the Reaction Curve obtained from the experimental results tabulated above (Tables A & B). It is composed of (a) an induction period (b) a constant rate section, and (c) a period AB which shows linear decrease in reaction rate as the oxidation proceeds. This linear relationship has been shown to continue until 90% at least of the total oxidation has occurred.

Extremely slow rates in the region of B in all cases forced reaction to be discontinued, but because of this relationship obtaining in the section AB, the line could be reasonably extrapolated to cut the rate axis at X. The value OX gave the oxygen uptake for the reaction at zero rate i.e. the total uptake, providing the film was completely illuminated.

Further Experimental details.

When the pointer had travelled so far that it was no//

no longer in focus in the telescope eyepiece, it could be returned to approximately its original position by reducing the pressure in the gauge jacket very slightly, without discontinuing the experiment. If the photo-oxidation had not proceeded far enough in one day, the system was allowed to stand in the dark overnight, with taps T_1 T_2 T_3 open, to prevent the possible fracture of the gauge due to unequal cooling of the gauge jacket and reaction vessel. After constant temperature had been achieved again on the following day, the taps were closed once more and illumination continued as before.

The addition of carbon dioxide gas could be achieved quite simply during illumination, merely by evacuating the lead between T_5 and T_6, filling with carbon dioxide to a pressure a few millimetres in excess of the original, and then opening T_1 and T_3 and allowing the gas to flow in until the desired pressure registered on M_1. Hydrogen could be admitted in the same way.

When adding water vapour however, it was necessary first to evacuate the system before admitting the vapour from the container H directly to the vessel and gauge only, and balancing the pressure by adding dry air to the gauge jacket. With T_3 and T_1 closed, the lines were then evacuated until free of water vapour, and any other gases added as described previously.

Examination of reaction products.//

Examination of reaction products.

Following the illumination of a pigment film in the presence of oxygen, water vapour and carbon dioxide, it was customary to examine the gaseous products for peroxide and aldehyde reactions (33 and 35) adopting the following procedure:-

A little distilled water was placed in trap C which was then surrounded by a suitable refrigerant, such as cardice/acetone or liquid oxygen, and evacuated. The gases present in the system were pumped slowly out by the oil pump, any condensable vapours being retained in C. After complete evacuation, the mixture in C was allowed to melt slowly at room temperature, and then removed from the apparatus. The solution was tested with Schiff's reagent for aldehyde, and with acid dichromate or titanic sulphate for peroxide reactions (92).

Invariably the film itself was extracted with water on removal and the extract tested in a similar fashion.

Alternatively, a measured volume of acetone was added to dissolve the pigment from the thallous bromide, and the absorption spectrum of the solution examined.

EXPERIMENTAL RESULTS.

EXPERIMENTAL RESULTS.

Examination of the first chlorophyll preparation.

In the first instance, it was considered a necessary step to confirm and examine further certain essential points of the early experiments carried out on this preparation. This work is summarised below under the heading "Normal Oxidations".

Normal Oxidations.

The following films were prepared on thallous bromide, as described previously and illuminated completely in the presence of 100 m.m. of oxygen by the "white" light from the Osira lamp. The temperature was maintained at 25°C throughout all experiments.

(1) Chlorophyll - carotenoid mixture.

Conditions:

0.3 grams thallous bromide

3.35 mls. chlorophyll-carotenoid solution 1.126 grams/litre in acetone.

Vessel + gauge volume = 85.2 ml.

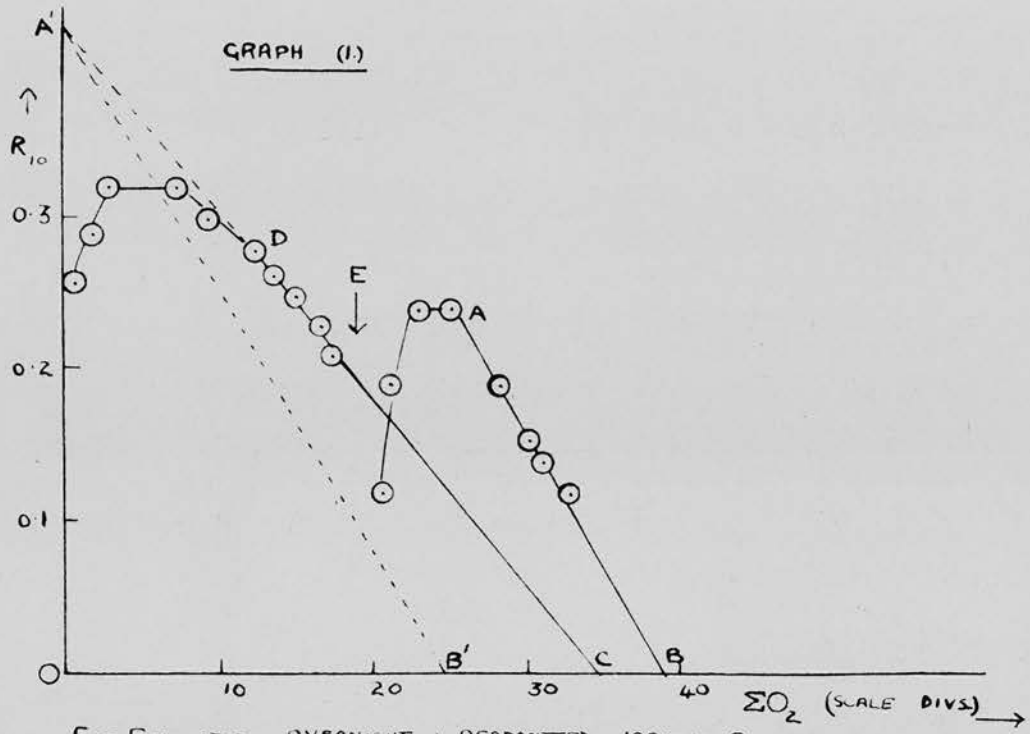
Results. ΣO_2 represents oxygen uptake, in scale divisions, at any point.

R_{10} represents rate, in scale divisions per 10 minutes, at the same point.

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.5	0.26	12.5	0.28	21.4	0.18
1.8	0.29	14.2	0.26	23.0	0.24
2.8	0.32	15.4	0.25	25.0	0.24
4.0	0.32	16.6	0.23	28.3	0.19
7.4	0.32	18.1	0.21	30.2	0.15
9.4	0.30	19.5	0.19	31.0	0.14
		20.7	0.14	32.6	0.12

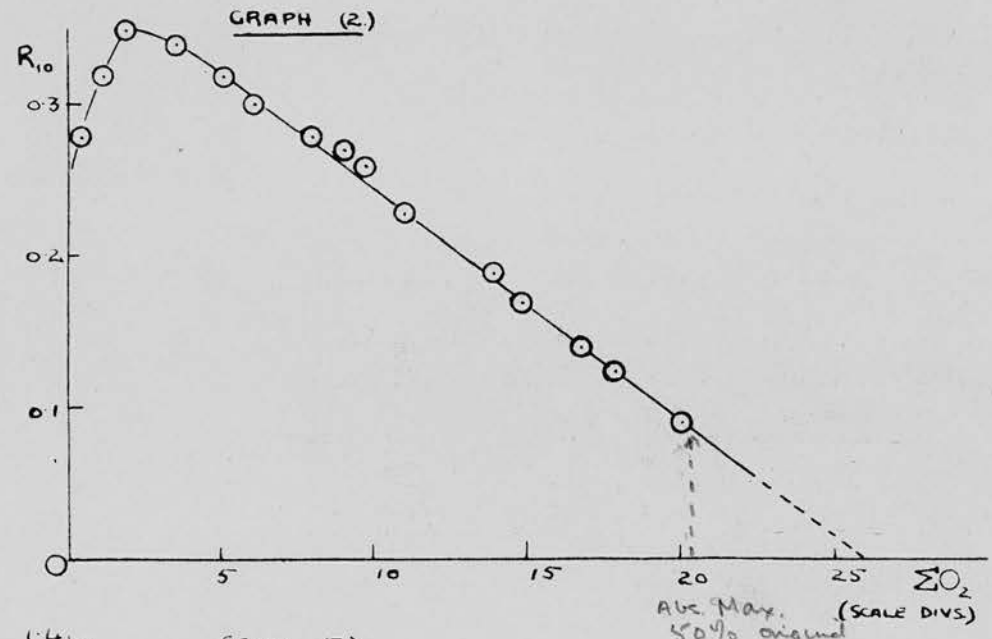
Total//

GRAPH (1.)



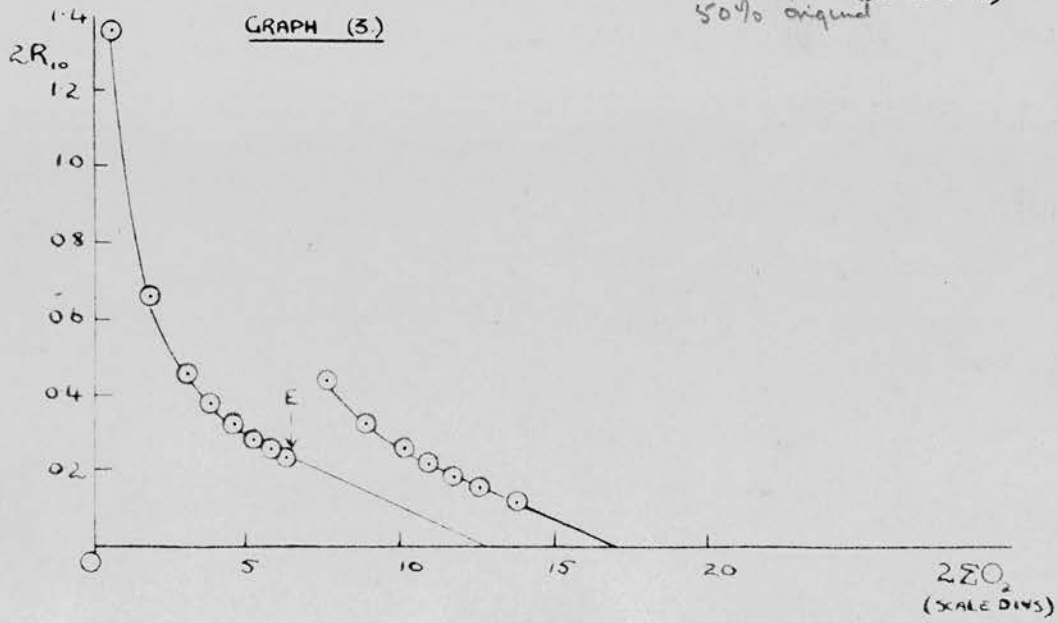
E = EVACUATED OVERNIGHT ; READMITTED 100 m.m. O_2.

GRAPH (2.)



Ave. Max. 50% original

GRAPH (3.)



Total ΣO_2, i.e. length OC on GRAPH (1), obtained by extrapolating the rate curve to R_{10} = 0, ... = 34.5 scale divisions.

A portion of the original stock solution, from which the above solution had been prepared by suitable dilution, was separated into its green and yellow components by a bonemeal column. The two fractions were examined as follows:-

(2) Chlorophyll Fraction.

Conditions.

0.3 grams thallous bromide.

3.35 mls. chlorophyll solution 0.92 grams/litre in acetone.

Vessel + gauge volume = 85.2 mls.

Results.

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.5	0.28	6.3	0.30	13.9	0.19
1.1	0.32	8.0	0.28	14.9	0.17
1.8	0.35	9.1	0.27	16.8	0.14
3.5	0.34	9.7	0.26	17.8	0.125
5.1	0.32	11.2	0.23	20.2	0.09

Total ΣO_2 = 25.9 scale divisions.

(3) Carotenoid Fraction.

Conditions:

0.3 grams thallous bromide.

3.35 mls. carotenoid solution 0.156 grams/litre in acetone.

Vessel + gauge volume = 86.9 ml.

Results//

Results.

$2\Sigma O_2$	$2R_{10}$	$2\Sigma O_2$	$2R_{10}$	$2\Sigma O_2$	$2R_{10}$
0.70	1.28	5.26	0.29	10.04	0.26
1.96	0.66	5.78	0.26	10.94	0.22
3.06	0.45	6.24	0.23	11.74	0.19
3.8	0.38	7.56	0.44	12.54	0.16
4.64	0.32	8.38	0.32	13.76	0.12

Total ΣO_2 = 6.3 scale divisions.

Analysis of Results.

The calculation of the molecular ratio of oxygen : pigment will be given in detail for the above experiments, but, to avoid repetition, the method will be assumed in dealing with later results which are similar in type.

Considering first the results for the chlorophyll fractions:-

A film prepared from a solution of chlorophyll, of concentration 0.92 grams/litre, was completely illuminated, a nd the total oxygen uptake was 25.9 scale divisions, i.e. 25.9 X 0.031 m.m.

Now, 22,400 ml. oxygen, at 760 m.m. pressure and a temperature of 0°C, ≡ 1 gram molecule.

∴ 85.2 ml. oxygen at (25.9 x 0.031) m.m. and 25°C

$$\equiv \frac{85.2 \times 273 \times 25.9 \times 0.031}{22400 \times 298 \times 760} \text{ gram molecules.}$$

Molecular weight of chlorophyll.

The bonemeal purification was designed to separate chlorophyll//

chlorophyll from carotenoids, no attempt being made to separate the chlorophyll fraction into the two very similar components, chlorophylls a and b. Consequently, the molecular weight of chlorophyll used in these calculations was taken as an approximate average of the two (893 and 907) and assumed to equal 900 (93).

Hence, $\frac{\text{Number of molecules of oxygen}}{\text{Number of molecules of chlorophyll}} = \frac{85.2 \times 25.9 \times 0.031 \times 273 \times 900 \times 1000}{22400 \times 760 \times 893 \times 0.92 \times 335}$

$= \underline{1.07}$

It was found that altering the average molecular weight used in this calculation to that of either of the two chlorophylls does not produce any significant change in this ratio.

Following similar reasoning, the molecular ratio of oxygen/carotenoids was obtained from the results of experiment (3) :-

Molecular weight of carotenoid fraction.

The two most common components of the "yellow fractions" obtained from plant extracts are:- xanthophyll $C_{40} H_{54} (OH)_2$, molecular weight 568, also known as lutein or luteol, and β-carotene $C_{40} H_{56}$, molecular weight 536. Most of the available data (94) indicate that in higher plants the average ratio by weight of xanthophyll: carotene = 4:1 to 6:1. The 'composite molecular weight' of the mixture will therefore be very close to that of xanthophyll, and, for//

for the present purposes, can be taken as 568.

A test, quoted by Spoehr (95) to recognise xanthophyll by the appearance of a green colour on dissolving in ethyl alcohol containing concentrated hydrochloric acid, was applied to the carotenoid fraction and the result showed that xanthophyll was definitely present. Furthermore, the fraction was soluble to some extent in formic acid, and could be extracted from petroleum ether solution by methyl alcohol. Both these properties are characteristic of xanthophyll (96).

$$\text{Hence, } \frac{\text{number of molecules of oxygen}}{\text{number of molecules of carotenoid}} = \frac{86.9 \times 6.3 \times 0.031 \times 273 \times 1000 \times 568}{22400 \times 760 \times 298 \times 0.156 \times 3.35}$$

$$= \underline{0.99}$$

The accuracy of these results depends on the determination of the gauge sensitivity; the maximum error involved in this measurement has already been reported as 3%. However, even allowing for this, the molecular ratios in both cases are sufficiently near unity to permit the assumption that, under these conditions, chlorophyll and carotenoids absorb one molecule of oxygen per molecule of pigment.

Having established this, and assuming that this unit molecular ratio holds for mixtures also, it is now possible to give a satisfactory interpretation of the original experiment, summarised in Graph (1), and to obtain a value for the ratio of chlorophyll to carotenoid present//

present in the mixture, from the following calculations:-

Let x represent the number of grams of chlorophyll present in Experiment I

y represent the number of grams of carotenoid present in Experiment I

Then $x + y = \frac{1.126}{1000} \times 3.35 = 377.1 \times 10^{-5}$ ..... (1)

and $\frac{x}{900} + \frac{y}{565} = \frac{34.5 \times 85.2 \times 0.031 \times 273}{22400 \times 760 \times 298} = 0.4904 \times 10^{-5}$

i.e. $142x + 225y = 0.4904 \times 4 \times 225 \times 142 \times 10^{-5}$ ... (2)

Multiply (1) by 142:-

$142x + 142y = 377.1 \times 142 \times 10^{-5}$ ..... (3)

Subtract (3) from (2)

$$83y = 10^{-5} \times 142 \{0.4904 \times 4 \times 225 - 377.1\}$$

$$\therefore y = \frac{10^{-5} \times 142 \times 64.3}{83} = 110.0 \times 10^{-5}$$

$$\therefore \%y = \frac{110.0}{377.1} \times 100 = 29.2$$

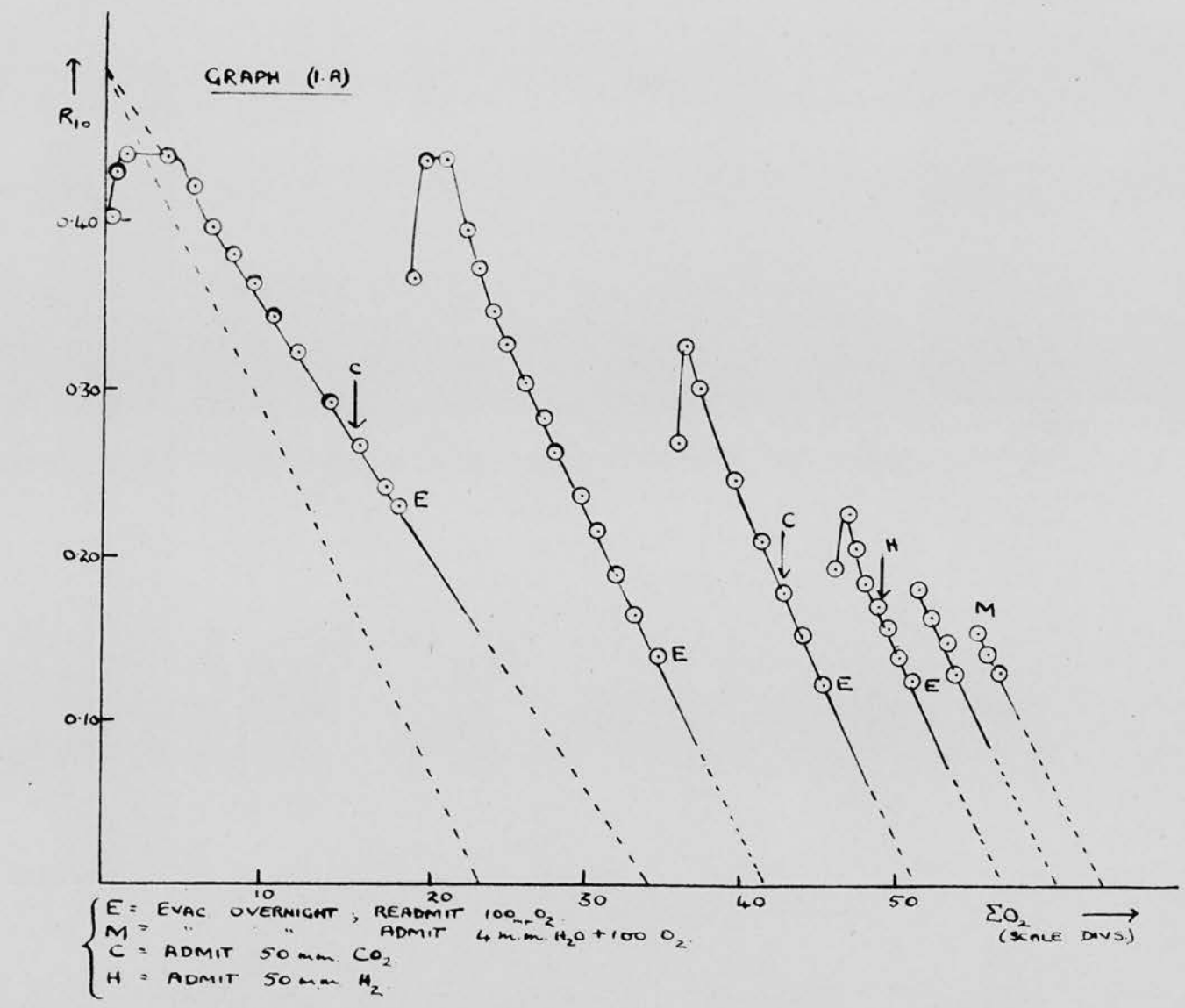
Hence $\%x = \underline{70.8}$.

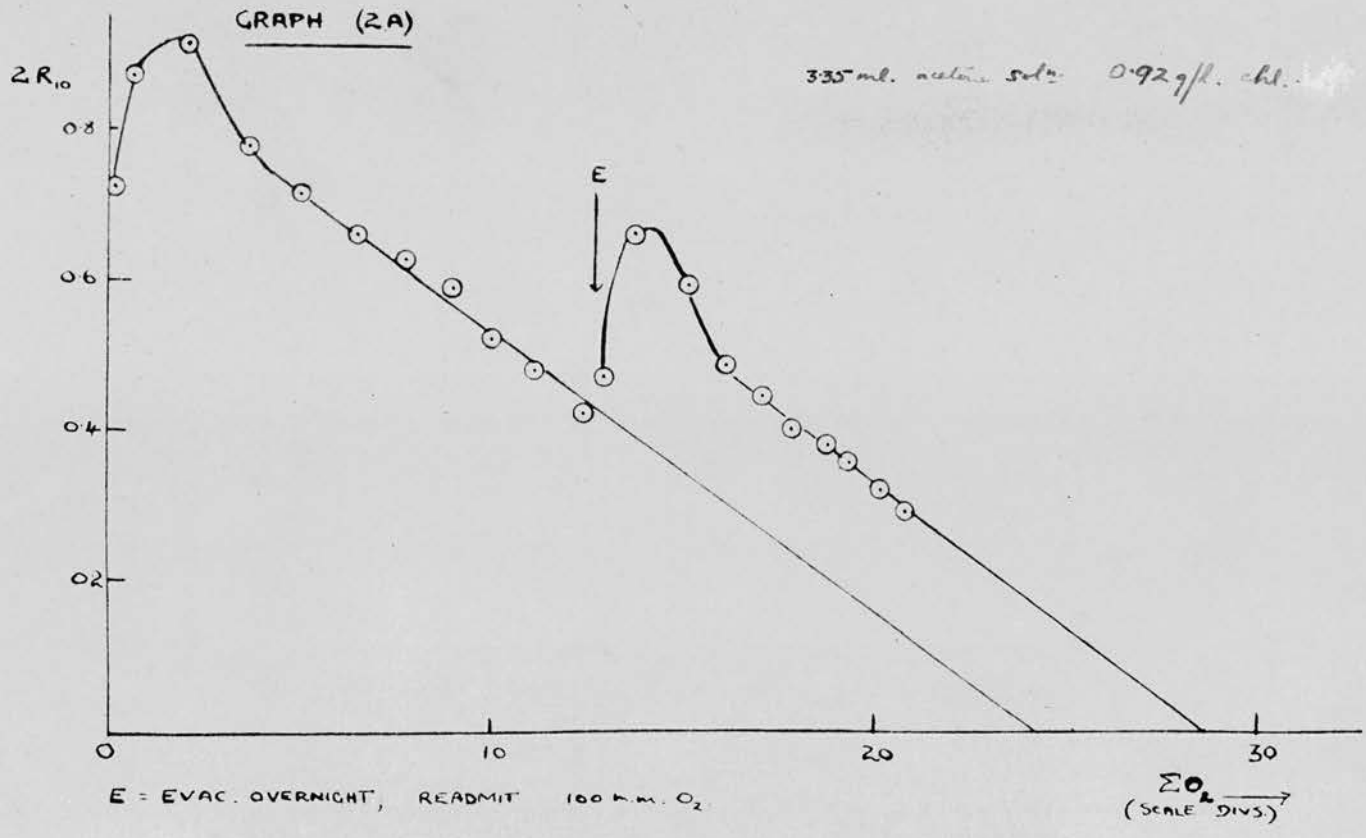
This result agrees reasonably with the value of 25% carotenoid obtained by weighing the fractions from a typical chromatogram, the latter figure being of course a minimum value because of residual adsorption on the column.

Discussion of the reaction curves obtained.

For purposes of comparison, a selection of the graphs of the 'normal oxidation' of chlorophyll on thallous bromide, obtained previously (32), are also reproduced here (Graphs 1A and 2A), and are included in the discussion.

(a) General form of the curve//


GRAPH (1.A)
R_{10}
0·40
0·30
0·20
0·10
C
E
E
C
E
H
E
M
10
20
30
40
50
ΣO_2
(SCALE DIVS.)
E = EVAC OVERNIGHT, READMIT 100 m.m. O_2
M = " " ADMIT 4 m.m. H_2O + 100 O_2
C = ADMIT 50 m.m. CO_2
H = ADMIT 50 m.m. H_2


GRAPH (2.A)
3·35 ml. acetic soln. 0·92 g/l. chl.
$2R_{10}$
0·8
0·6
0·4
0·2
E
0
10
20
30
ΣO_2
(SCALE DIVS.)
E = EVAC. OVERNIGHT, READMIT 100 m.m. O_2

General form of the curve.

From a preliminary examination of these curves it is obvious that they conform to the type generally obtained in auto-oxidations (91), being composed of:-

(a) an induction or acceleration period.

(b) a constant rate period (in types 1 & 1A only).

(c) a period in which rates fall off linearly with the extent of oxidation.

An additional point of resemblance is that one molecule of oxygen is taken up per molecule of oxidisable substance.

(b) Reversibility.

At the points indicated on the various graphs, the system was evacuated, and on re-admitting 100 m.m. of oxygen and continuing the illumination, it was obvious that some reversal had occurred. In the case of Experiments (2) and (3), the linear parts of the curve obtained after evacuation were all parallel to the original, but in the very first experiment, the slope of this second section was slightly steeper than the original. Repeated evacuation gave repetitions of this latter slope.

This effect can be explained when one considers the relative extents to which each fraction can be reversed. It is obvious from Graph (3) that the oxidation of carotenoids can be reversed to only a small degree. In addition, this reaction has a relatively fast initial rate. Consequently, the slope//

slope AB on graph (1) can be ascribed, with some justification, almost entirely to chlorophyll oxidation. This gains further support from the fact that the length OB' corresponds to unit oxygen:chlorophyll molecular ratio.

Incidentally this provides another method of obtaining a value for the chlorophyll/carotenoid ratio, thus:- Produce CD to cut the rate axis at A' and from this point draw A'B' parallel to AB. If the lengths OB' and OC are measured, their ratio is found to give a value for the percentage chlorophyll in the mixture

$$\frac{OB'}{OC} \times 100 = \frac{24.5}{34.5} \times 100 = \underline{71.0\%}$$

The oxide could not however be reversed in this way indefinitely. The decrease in the extent of the reversion at advanced stages in the reaction indicated the gradual formation of an irreversible form of the product.

(c) Effect of carbon dioxide, water vapour and hydrogen.

At the points indicated on the graphs, separate additions of the above gases were made to the system, followed by the usual 100 m.m. of oxygen. In no instance was any significant effect observed on the rate of pressure decrease, or on the extrapolated value for the total gas uptake when the film was illuminated under the same conditions as before. In adding water vapour, it was necessary first to evacuate//

evacuate the system and consequently, the usual reversal took place, but the slope of the curve obtained did not differ from that obtained in the presence of oxygen alone. Tests on the gases frozen out at the end of an experiment such as this gave no evidence of peroxide or aldehyde reaction (cf.33). The same was true of any aqueous extract of the resulting film, i.e. no water-soluble peroxide is formed in detectable amounts.

(d) Variation of rate with light intensity.

When the intensity of the incident light was reduced by the insertion of neutral grey filters, the rates obtained were found to be directly proportional to the percentage transmission.

(e) Variation of rate with the wavelength of the incident light.

It was found that the rates of oxidation were exceptionally slow in red light. It was possible that even this slow reaction was due to the small amount of light of shorter wave-length passed by the filter.

The large increase in rate with Osira "white light" was regarded as due to the initial absorption by thallous bromide, the reaction being then a photo-oxidation sensitised by thallous bromide.

The shape of the reaction curves.

From a comparison of graphs of types (1) and (2), it was obvious that the "constant rate portion" occurred//

occurred only with films of mixed pigments, and was therefore to be connected with the presence of carotenoids. Graph (3) however, obtained with a film of carotenoids alone, of approximately half the weight of yellow pigment present in the mixed films, did not show this constant rate period.

The question was therefore investigated further by preparing a series of films on thallous bromide, in which firstly, the total amount of mixed pigments, and secondly, the proportions by weight of the two pigments were varied.

It was found that the extent of the constant rate period was independent of the amount of chlorophyll but varied directly with the weight of carotenoids, over the range chosen.

Summary of results.

(a) Mixed Films.

%age carotenoid by weight	Actual weight of pigments. Ch.	Car.	l. (ℓ)
15	2.7mg	0.48	1.0
30	2.67	1.13	4.5
	1.34	0.57	2.5
	0.65	0.28	1.0
	5.36	2.28	9.0
60	2.7	3.76	15.5
90	0.45	3.76	16.0
-	3.07	-	0.0.
100	-	0.47	1.0
		1.88	7.5
		3.76	16.0

(b) Pure Carotene Films.

l = length of the straight line period in scale divisions.

The length of the straight line period is difficult to estimate accurately when exceptionally small amounts//

amounts of carotene are used in preparing the film. In fact, no definite existence could be claimed for this period unless amounts greater than 0.5 milligrams were present. However, with larger amounts of pigment, producing correspondingly longer straight line periods, $\underline{l}$ can be estimated to within $\pm$ 0.5 scale divisions. It is therefore clear why no constant rate period was observed with the original film (Experiment 3), since the amount of carotenoid used was hardly sufficient to show a straight line of any definite length.

The extent of the constant rate period was independent of the method of evacuating the system i.e. whether by oil or mercury diffusion pump. In every case the apparatus was pumped out for a minimum of one to two hours, using a liquid oxygen trap, and then washed out several times with small amounts of oxygen gas. After standing evacuated overnight, the pumping and washing out were repeated before admitting oxygen and commencing the run.

In one experiment, after brief evacuation at the end of the constant rate period, 10 scale divisions of acetone vapour were added with 100 m.m. of oxygen. The acetone had no effect on the progressively decreasing rates normally obtained at this stage. It therefore seemed obvious that the constant rate period was not due, as had been suspected, especially in the thicker films, to any residual solvent vapour, trapped//

trapped in the film.

In the experiment with 1.88 mg. of pure carotenoids, evacuation overnight was applied once the rates had reached the decreasing stage. On re-admitting oxygen it was found that "1" could be repeated, although diminished slightly in extent.

Furthermore, with these thicker films, the final (extrapolated) value for the oxygen uptake gave again a unit molecular ratio of oxygen to carotenoid.

Since the constant rate period appears in the initial stages of the oxidation of the mixed films, it would appear that carotenoids either start to oxidise before the chlorophyll portion, or that oxidation is here essentially controlled by the carotenoids present. It was also observed that films of pure carotenoid oxidise more rapidly than those of pure chlorophyll, the latter generally showing quite an induction, or acceleration, period.

Interpretation of results.

It is not intended to give a detailed interpretation at this stage, but rather to present just enough argument to give a working hypothesis, and to reserve further deductions until the results of all three chlorophyll preparations can be considered together.

Since the photo-oxidation of chlorophyll has been shown to have many features in common with the auto-oxidation of organic molecules in general, it is reasonable//

reasonable to suppose that the oxygen: pigment molecular ratio of unity indicates peroxide formation in this instance also. The location of the position of oxidation presents some difficulty, as it can not be deduced directly from the foregoing results. In the combustion of paraffins, the point of oxidative attack is generally the tertiary - CH group (97), but the direct removal of such a hydrogen atom required more energy than is supplied under the present conditions. However, the properties of chlorophyll solutions (88) indicate that the hydrogen atom on the tertiary carbon atom, C_{10}, is in a particularly reactive state; it is at this position then, that the reversible oxide is visualised as being formed in these experiments detailed above.

As regards the carotenoid fraction, according to Rabinowitch (98), the oxidation of such compounds results in the formation of double bond peroxides. The results obtained, however, indicate that, under the present conditions, auto-oxidation occurs at only one of these bonds.

The mechanism of such oxidations will be discussed in detail at a later stage.

Oxidation of the first chlorophyll sample in the presence of certain acids.

In introducing these acids to the system it was hoped originally they would facilitate an uptake of carbon dioxide and its possible transformation into some//

some photosynthesised product. This, it may be recalled, had never been detected in the early work on the films of pigment alone.

The first to be chosen for study was phosphoric acid which, because of the close connection of organic phosphates with photosynthetic schemes, and the ability of the inorganic phosphates in leaves to absorb quantities of carbon dioxide (99), seemed to offer promise of interesting results.

The other acids, pyruvic and ascorbic were selected partly to achieve the same effect, the former especially as it is concerned in certain carboxylation cycles, and also to ascertain whether the effects of such naturally occuring acids were comparable with one of inorganic origin.

In every case the amount of acid added to the pigment film was such as to give a molecular ratio of pigment to acid of unity.

The other conditions of oxidation were maintained as before i.e. 100 m.m. oxygen - Osira light - and a temperature of 25°C.

The possible effect of light on the acid itself was first investigated in each case by depositing exactly the same amount of acid solution as would be used in the later experiments directly on to 0.3 grams thallous bromide in the reaction vessel and evaporating and evacuating in the normal way.

These//

These films were then illuminated with the same Osira Light -

1. in vacuo
2. in the presence of 100 m.m. of oxygen.
3. in the presence of 100 m.m. of carbon dioxide.
4. in the presence of 1 m.m. water vapour, 10 m.m. carbon dioxide, and 100 m.m. of oxygen.

(No.4. applies to phosphoric acid only)

No significant pressure changes were recorded in any case.

Phosphoric Acid Experiments.

(4) chlorophyll-carotenoid mixture.

Conditions.

0.3 gram thallous bromide.

3.38 ml. chlorophyll-carotenoid solution 1.144 grams/litre in acetone.

1.38 ml. phosphoric acid 0.303 grams/litre in water.

Vessel + gauge volume = 86.9 ml.

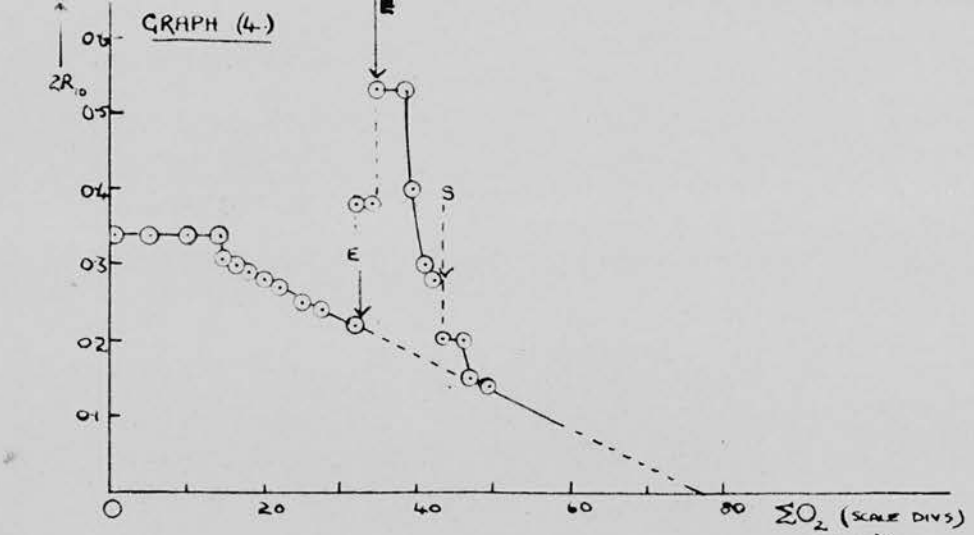
Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.1	0.34	18.0	0.29	28.6	0.25	39.7	0.40.
5.0	0.34	20.0	0.28	32.0	0.22	41.1	0.30
10.0	0.34	22.0	0.27	32.05	0.38	42.2	0.28
14.2	0.34	24.0	0.26	34.9	0.38	43.15	0.20
14.7	0.31	25.0	0.25	35.0	0.53	46.35	0.20
16.5	0.30	27.5	0.24	38.6	0.53	46.75	0.15
						49.0	0.14

Total ΣO_2 = 77.2 scale divisions.

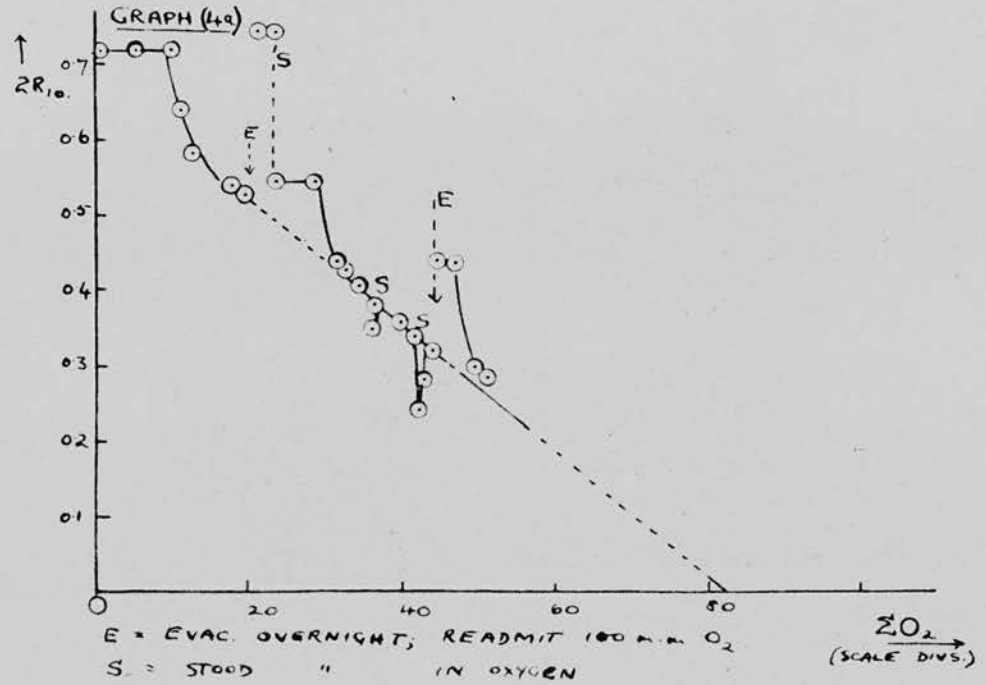
(5) Chlorophyll Fraction.

Conditions:



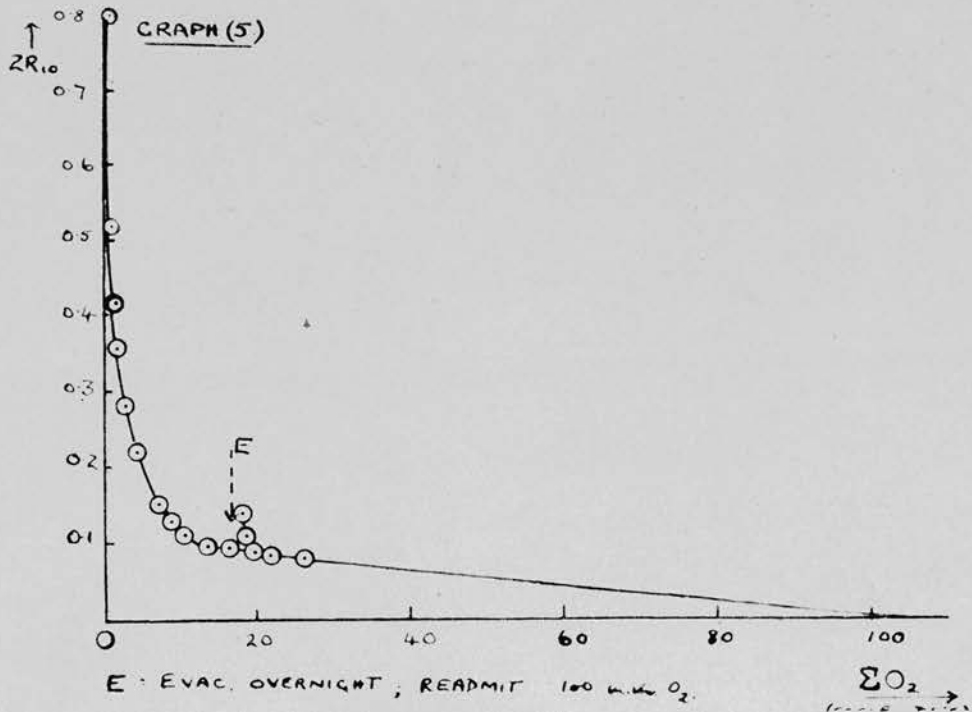
E = EVAC ; ADMITTED 6 m.m H_2O, 50 m.m CO_2 , 100 m.m O_2

S = STOOD IN OXYGEN OVERNIGHT.



E = EVAC. OVERNIGHT; READMIT 100 m.m O_2

S = STOOD " IN OXYGEN



E = EVAC. OVERNIGHT ; READMIT 100 m.m O_2.

Conditions:

0.3 gram thallous bromide.

1.425 ml. chlorophyll solution (bonemeal purified) 3.16 grams/litre in acetone.

1.49 ml. phosphoric acid 0.3287 grams/litre in water.

Vessel + gauge volume = 86.9 ml.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.3	0.80	4.8	0.22	13.3	0.095	19.7	0.09
0.7	0.52	7.3	0.15	16.5	0.09	20.4	0.085
1.1	0.42	8.7	0.13	17.5	0.10	21.7	0.085
1.9	0.36	10.2	0.11	18.0	0.14	26.0	0.08
3.0	0.28	11.5	0.10	18.7	0.12		

Total ΣO_2 = 104.5 scale divisions.

$\frac{\text{oxygen}}{\text{chlorophyll}}$ = 3.03 molecularly.

This experiment was repeated and the result confirmed.

In both these experiments, (4) and (5), no reversibility on evacuation was observed, the rates although increased temporarily, rejoining the original curve eventually. In the former experiment, carbon dioxide and water vapour were always present after evacuation. The experiment was therefore repeated and the non-reversibility shown to be independent of the presence of carbon dioxide and water vapour. (These results are presented in Graph (4a).)

(6) Carotenoid Fraction.

Conditions://

Conditions:

0.3 gram thallous bromide.

3.35 ml. carotenoid solution 0.148 Grams/litre in acetone.

0.29 ml. phosphoric acid aqueous solution, 0.2936 g/l.

Vessel + gauge volume = 85.2 ml.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.40	0.81	4.15	0.20	6.60	0.16
1.10	0.59	4.55	0.17	6.80	0.14
1.62	0.44	4.88	0.14	7.20	0.11
2.22	0.36	5.18	0.12	7.40	0.09
2.53	0.32	5.76	0.28	7.60	0.08

Total ΣO_2 = 6.6 scale divisions (extrapolated value)

$\frac{\text{oxygen}}{\text{carotenoid.}}$ = 1.08 molecularly

When evacuation was applied (at ΣO_2 = 5.3) some reversion was again evident.

It was obvious from the result of the above experiment that the acid had no effect whatever on the oxidation of carotenoids. The graph had exactly the same form as that of Experiment (3).

We may therefore assume that in Experiment (4), the oxygen/carotenoid ratio is again unity, and so obtain a value for the amount of oxygen absorbed by this component of the mixture, present to the extent of 29.2% by weight, thus:-

$$\frac{\text{number of molecules of oxygen}}{\text{number of molecules of carotenoid}} = 1 =$$

$$\frac{y \times 86.9 \times 568 \times 0.031 \times 273 \times 1000 \times 100}{22400 \times 760 \times 298 \times 1.144 \times 3.35 \times 29.2}$$

Where y = number of scale divisions of oxygen taken up by the carotenoids.

Hence y = 13.58 scale divisions.

∴ Oxygen uptake due to chlorophyll present =

= 77.2 - 13.6

= 63.6 scale divisions.

$$\therefore \frac{\text{oxygen}}{\text{chlorophyll}} = 3.05 \text{ molecularly.}$$

With these acid films, the rates of oxidation were somewhat reduced, especially in the case of "purified" chlorophyll. The mixed pigments however still oxidised at a reasonable rate, and it was therefore decided to use this solution in further acid experiments.

(7) Pyruvic Acid Experiments.

Conditions:

0.3 grams thallous bromide.

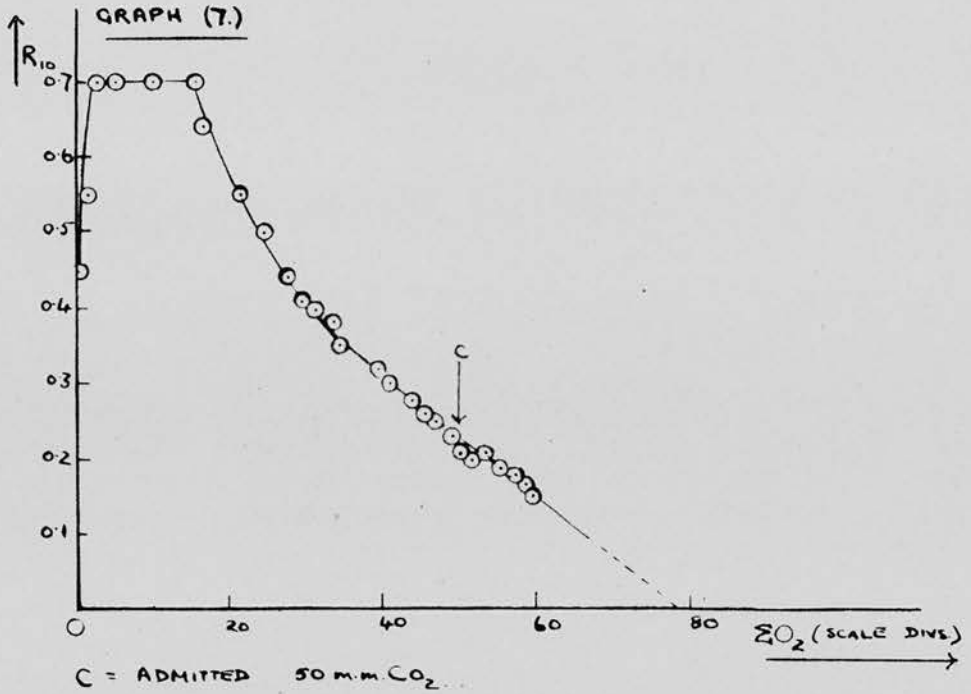
3.35 ml. chlorophyll-carotenoid solution, 1.144 g./l. in acetone.

1.01 ml. pyruvic acid, aqueous solution, 0.278 g./l.

Vessel + gauge volume = 86.9 ml.

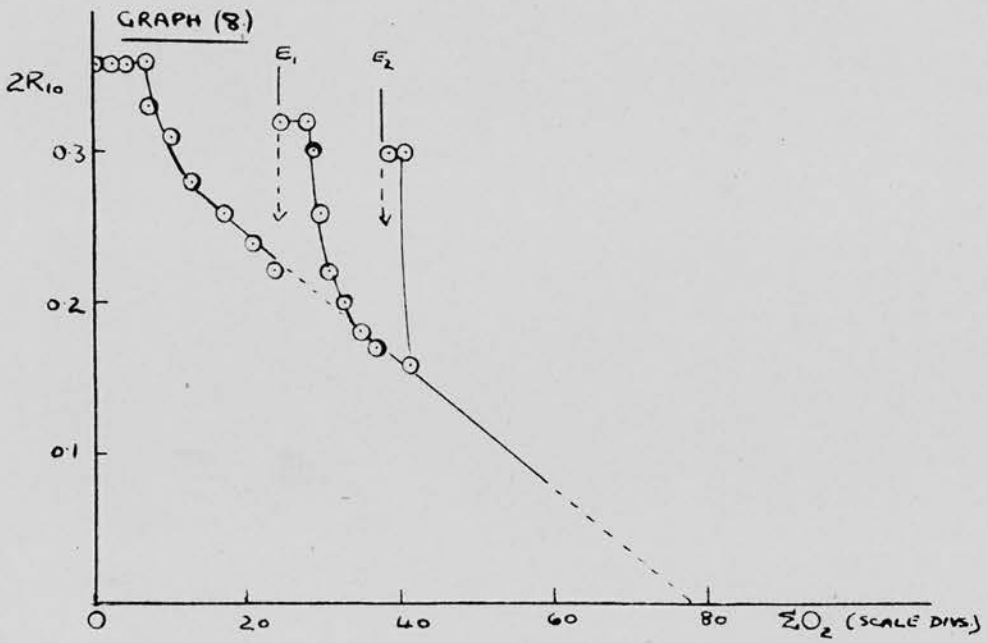
Results.

GRAPH (7.)



C = ADMITTED 50 m.m. CO_2.

GRAPH (8.)



E_1 = EVACUATED OVERNIGHT; READMITTED 100 m.m. O_2.

E_2 = " " ; ADMITTED 2 m.m. H_2O, 50 m.m. CO_2 + 100 m.m. O_2.

Results.

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.8	0.45	18.7	0.64	33.5	0.38	45.3	0.26	55.2	0.19
1.7	0.55	21.2	0.55	37.1	0.35	46.7	0.25	57.1	0.18
2.4	0.70	24.3	0.50	39.1	0.32	49.0	0.23	58.4	0.17
5.0	0.70	27.6	0.44	40.9	0.30	50.0	0.21	59.3	0.15
10.0	0.70	29.7	0.41	42.1	0.29	51.4	0.20		
17.7	0.70	31.0	0.40	43.7	0.28	53.0	0.21		

Total ΣO_2 = 78.5 scale divisions.

ΣO_2 due to chlorophyll = 78.5 - 13.6 = 64.9 scale divisions.

$\therefore \frac{\text{oxygen}}{\text{chlorophyll}}$ = 3.11 molecularly.

A second similar experiment was carried out to confirm the results of (7), and to examine the effect of carbon dioxide gas and water vapour on the reaction. The oxygen/chlorophyll molecular ratio obtained in this case was 3.05.

Since it was believed that the order of deposition of chlorophyll and acid on the thallous bromide might affect the reaction, a third experiment was performed, using an alcoholic solution of acid. With an aqueous solution of acid, in which both chlorophyll and carotenoid are insoluble, the method of preparation results in the deposition of an acid layer on top of the pigment. In this experiment, by contrast, an intimately mixed film was obtained. The result was exactly the same however, no matter which method of preparation was used.

A final pyruvic acid film was prepared under the following//

following conditions:-

0.3 gram thallous bromide.

3.35 ml. chlorophyll/carotenoid solution 1.126 grams/litre.

0.48 ml. phosphoric acid, aqueous solution, 0.309 grams/litre.

0.49 ml. pyruvic acid, aqueous solution, 0.270 grams/litre.

Vessel + gauge volume = 85.2 ml.

Total ΣO_2 = 77.0 scale divisions.

$\therefore \frac{\text{Oxygen}}{\text{chlorophyll}}$ = 3.02 molecularly.

This film of mixed acids, therefore, gave the same result as before. Very small amounts (5 - 10 scale divisions) of carbon dioxide were added to the system at one stage, to see whether any adsorption, in the dark or on illumination, could be detected at low pressures.

(8) Ascorbic Acid Experiments.

Conditions:

0.3 grams thallous bromide.

3.35 ml. chlorophyll-carotenoid solution 1.144 grams/litre.

1.0 ml. ascorbic acid, aqueous solution, 0.563 grams/litre.

Vessel + gauge volume = 86.9 ml.

Results.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.1	0.36	17.0	0.26	29.3	0.26
2.0	0.36	17.8	0.25	30.7	0.22
4.0	0.36	20.6	0.24	32.4	0.20
6.5	0.36	23.6	0.22	34.9	0.18
7.3	0.33	24.0	0.32	36.8	0.17
10.0	0.31	27.8	0.32	38.5	0.30
12.5	0.28	28.4	0.30	40.2	0.30
				41.5	0.16

Total ΣO_2 = 77.6 scale divisions.

ΣO_2 due to chlorophyll present = 64.0 scale divisions.

$\therefore \frac{\text{oxygen}}{\text{chlorophyll}}$ = 3.08. molecularly.

Variation of rate with light intensity.

In certain experiments in this series the intensity of the incident light was reduced by the use of neutral glass filters. Again, it was observed that the rates so obtained were proportional to the transmitted light.

Effect of carbon dioxide and water vapour.

(1) At the points indicated on the various graphs, carbon dioxide was admitted to the system without interrupting the illumination. It was found that the presence of this gas had no effect either on the rates at which the pressure of the system decreased, or on the total pressure decrease. It was therefore concluded that under these conditions, carbon dioxide was not being adsorbed by or reacting with the chlorophyll//

chlorophyll present, although the results could also be explained, with reference to the chlorophyll molecule, by the entry of a molecule of carbon dioxide and the simultaneous expulsion of an oxygen molecule.

In the case of mixtures of carbon dioxide, water vapour and oxygen, apart from a temporary increase in rates caused most probably by the evacuation of the system before admitting water vapour, the normal rates of pressure decrease, due presumably to oxidation, were obtained as before.

(2) Tests on the film and on the gases present in the system at the end of such illumination, in this case also, yielded no evidence of peroxide or aldehyde properties which might be expected from any photosynthesised material (cf.33).

Discussion of the effects of acid on the oxidation of chlorophyll.

(a) Increase in oxygen uptake.

From an examination of these results, it is obvious that the total pressure decrease, obtained by extrapolating to zero rate, is in every case much greater than in the "normal oxidations". Furthermore, since Experiment (6) showed that the oxygen uptake of the carotenoid fraction is unaffected by these acids, this increase must be due to the chlorophyll component which is apparently absorbing a further two molecules of oxygen per molecule of pigment.

However, it is also possible that chlorophyll is acting//

acting as a photosensitiser for the oxidation of these acids. (The result of Experiment (6) makes it obvious that carotenoids do not act in this capacity.) The results of an additional experiment using $\frac{1}{50}$th of the amount of acid present in Experiment (4) enabled this possibility to be dismissed, for the following reasons:-

Experimental details:-

(9.) 0.3 grams thallous bromide

3.35 ml. chlorophyll-carotenoid solution 1.144 g/l.

1.38 ml. phosphoric acid, aqueous solution, $\frac{0.303}{50}$ g/l.

Vessel + gauge volume = 85.2 ml.

Total oxygen uptake (extrapolated) = 55.5 scale divisions.

Now, if in Experiment (4) for example, the acid itself was responsible for absorbing the additional oxygen, i.e. for approximately 77.2 - 35.0 = 42.2 scale divisions we could expect that in this experiment, the additional uptake would be $\frac{42.2}{50}$ scale divisions, giving a total uptake of 35.0 + 1.0 (approximately)

= 36.0 scale divisions.

By contrast, approximately 20 extra divisions of oxygen have to be accounted for in Experiment (9).

It would seem obvious therefore that the phosphoric acid cannot in itself be responsible for the increased uptake, but only indirectly through its effect on the chlorophyll.

Sensitised//

Sensitised auto-oxidations of pyruvic and ascorbic acids by alkaline chlorophyll and bacterio-chlorophyll respectively, have been reported in the literature (100); it was therefore important to consider the possibility of this happening under the present conditions. However, if we consider the pressure changes in the reaction :-

$$CH_3.CO.COOH + 2O_2 \longrightarrow \begin{matrix} COOH \\ | \\ COOH \end{matrix} + H_2O + CO_2$$

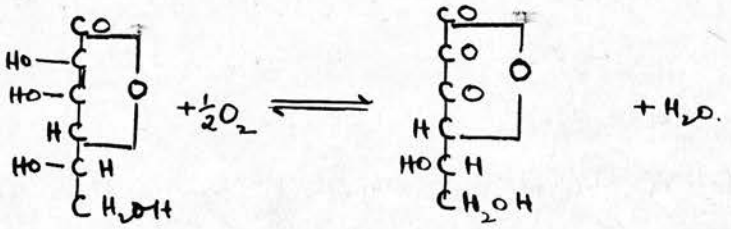
we have at the most, if the water vapour formed is completely adsorbed, a change of only one volume of gas. Therefore, with the simultaneous "normal oxidation" of chlorophyll, the oxygen uptake figures would give a 2:1 molecular ratio, this being a maximum value as some water would remain in the vapour phase.

If further oxidation of the oxalic acid occurs:-

$$2 \begin{matrix} COOH \\ | \\ COOH \end{matrix} + O_2 \longrightarrow 4CO_2 + 2H_2O$$

an increase in pressure would be observed.

Similarly, in the case of ascorbic acid:-



even with complete adsorption of the water vapour produced, the decrease is still only $\frac{1}{2}$ volume.

These//

These considerations, taken in conjunction with the fact that the effects produced are in the main independent of the actual acid used, indicate that the increased oxidation must be associated with some change in the chlorophyll molecule itself.

According to Rabinowitch and other authorities (101), the general effect of such acids on chlorophyll is to remove the central magnesium atom from the molecule, producing the compound known as pheophytin. Assuming that this happens here, as seems highly probable under the conditions of preparing the films, two further oxidisable centres will be produced at the point of detachment of the magnesium, giving in all, three positions of oxidative attack, and hence the uptake of three molecules of oxygen, with presumably the formation of a peroxide group in each instance. In this connection, it must be mentioned that the oxygen:pigment molecular ratio of 3:1 is not altered significantly by replacing the molecular weight of chlorophyll by that of pheophytin in the calculations.

The displacement of the co-ordinately bound magnesium atom from such a conjugated system is bound to restrict the resonance possibilities of the molecule. It is significant with regard tb this point that no reversibility can be detected with acid-containing films, the rates, in the case of phosphoric and ascorbic acids, showing a temporary increase//

increase on evacuation, only to rejoin the original straight line again fairly readily. With pyruvic acid, no increase in rates whatever was observed. This is most probably an outcome of the decreased stability of the metal-free molecule, and it will be discussed more fully after the examination of the next chlorophyll preparation.

The fact that such preparations of pheophytin in the solid state oxidise to a greater extent than chlorophyll itself provides a marked contrast with results obtained with solutions of the pigments. It has been suggested that the "allomerisation" (22) of chlorophyll solutions in alcohol consisted of the oxidation of the pigment at C_{10}. This has not been definitely established since the part played by the solvent has never been elucidated. However, it has been observed that pheophytin in alcohol solution could not be "allomerised", (22) and showed no oxygen uptake under these conditions.

If allomerisation can indeed be identified with an oxidation at C_{10}, the reactions of solid and dissolved pheophytin appear to be quite distinct, at least as regards auto-oxidation.

(b) Increase in the extent of the constant rate period.

In these acid experiments, it was again obvious that no constant rate period occurred in the absence of carotenoids. With films containing a mixture of the two pigments, "1" was two to three times as extensive//

extensive as normally, although, from the result of Experiment (6), the acid had no effect on the oxidation of carotenoids alone, the reversibility and the oxygen uptake being just as before. However, as again the amount of pigment used was very small and hardly sufficient to produce a straight line period under normal conditions, further films using twice this amount were prepared and acid added to give a unit molecular ratio of acid to pigment. It was found that, although the oxygen uptake remained unchanged, the constant rate period was indeed increased two to three-fold.

There is little in the literature relating to any effect of acid on carotenoids which would explain this increase in the constant rate period, except perhaps the fact that the treatment of carotenoid mixtures with acids, in the preparation of silage, leads to a decrease in the xanthophyll content and a corresponding increase in the amount of carotene present (102). Since "1" increases with increasing carotene content on acid treatment, it would appear from the experiments that it is the carotene portion of the "yellow fraction" which is responsible for the occurrence of the constant rate period, although it is difficult to see why such similar compounds as xanthophyll and carotene should differ in this respect.

Presence//

Presence of copper in the first chlorophyll sample.

According to the manufacturers' report, their original preparation had been "stabilised" by the addition of copper to the extent of 0.6%.

This was first of all confirmed (a) on the solid paste as supplied by the firm and (b) on a sample of the bonemeal-purified chlorophyll.

Results.

(a) Weight of crucible and chlorophyll paste = 21.8870 grams.
Weight of crucible alone = 20.8070 grams.

∴ weight of paste = 1.080 grams.

After ashing, this was dissolved in 1:1 nitric acid and diluted to 50 mls. To obtain a solution of suitable concentration, a portion of this original solution was diluted a further 10 times.

The standard solution contained $.01 \times \frac{.389}{.393}$ mg copper per ml.

It was found that:-

3.15 ml. of the diluted chlorophyll ash solution ≡ 5 mls. of the standard solution.

$$\equiv 5 \times .01 \times \frac{.389}{.393} \text{ mg. copper.}$$

∴ 50 mls. of the original ash solution

$$\equiv \frac{5 \times 01 \times .389 \times 50}{.393 \times 3.15}$$

$$= 7.85 \text{ mg. copper.}$$

∴ Percentage copper in the crude paste = $\frac{0.00785 \times 100}{1.080}$

$$= \underline{0.73}.$$

(b) weight//

(b) weight of crucible and bonemeal chlorophyll = 17.5631 grams.

weight of crucible alone = 17.5140 grams

∴ weight of chlorophyll = 0.0491 grams.

The ash from this sample was dissolved in 1:1 nitric acid and diluted to 50 mls.

The standard solution used in this case contained exactly 0.01 mg. copper per ml.

It was found that:-

10 ml. chlorophyll ash solution ≡ 7.1 ml. standard solution.

≡ 7.1 x 0.01 mg. copper

∴ 50 ml. ≡ 7.1 x 0.01 x 50

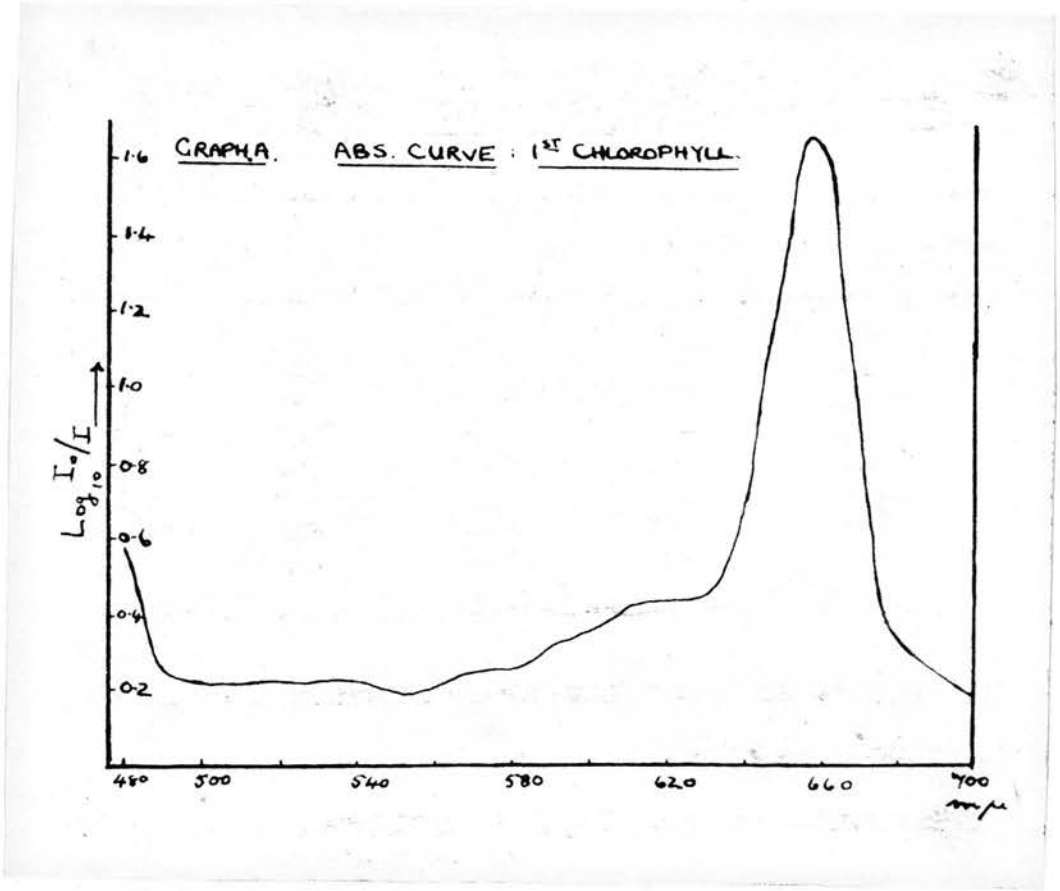
≡ 0.36 mg. copper.

∴ Percentage copper present in "pure" chlorophyll = $\frac{0.36}{49.1}$ x 100 = 0.73.

From these results it would appear that all the copper present is incorporated in the chlorophyll molecule, since it is not removed on purification.

The addition of copper as a stabiliser or colour preservative is a fairly common practice (103), the only obvious effects being the suppression of any fluorescence in ultra-violet light (104) and the inability of the copper-treated preparation to give the "phase reaction" (88). The absorption spectrum of this first sample however agreed reasonably well with that quoted by Rabinowitch for pure chlorophyll (24) :-

Results.//


GRAPH A. ABS. CURVE : 1ST CHLOROPHYLL.
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Log10 I0/I
480
500
540
580
620
660
700
mμ

Results.

Absorption Spectrum of the "first chlorophyll preparation" i.e. of copper-substituted chlorophyll.

λ (mμ)	Log_{10} I_0/I	λ	Log_{10} I_0/I	λ	Log_{10} I_0/I
700	0.20	630	0.43	550	0.20
690	0.24	620	0.44	540	0.23
680	0.32	610	0.43	530	0.23
670	0.93	600	0.35	520	0.22
660	1.65	590	0.33	510	0.23
655	1.65	580	0.25	500	0.22
650	1.34	570	0.25	490	0.23
640	0.75	560	0.20	480	0.58

The Log_{10} I_0/I values were obtained directly from the photometer drum readings.

I_0 = Intensity of the light transmitted by the solvent cell.

I = ditto transmitted by the solution cell.

C = concentration of the solution = 0.198 grams/litre.

l = thickness of the solution layer = 1.000 cms.

Owing to the decreased sensitivity of the eye to light of shorter wavelengths, the maximum in the blue region could not be determined. The maximum in the red was found to occur between 6550 and 6600 Å. Zscheile has shown (18) that in this region a straight line relationship obtains between concentration and $\log_{10}$ I_0/I for chlorophyll solutions within a certain range, which included the concentrations actually employed in this case.

The absorption curve of the oxidised product showed a distinct depression of the red maximum; for example//

example, the product of Experiment (2) gave a maximum at 6650 - 6600 Å of approximately one half the original value. Apart from this, the curves were essentially similar in shape (cf. 24). However, since it has been reported that the red maximum can be depressed to a certain extent merely by adsorbing chlorophyll on certain solids (18), it was important to see how much was due to this cause in the present case. The absorption curve of the same solution was therefore determined after adsorption on thallous bromide in a crystallising dish. It was found that only a very slight depression, approximately 3% at the red maximum occurred after such treatment. The absorption curve of the product from Experiment (5), presumably oxidised phoophytin, showed not only this depression of the red maximum, but also a shift in its position to longer wavelengths (6650 Å), and the appearance of small bands at 5050 and 5350Å.

According to Rabinowitch (101) the central magnesium atom of chlorophyll can be replaced readily by other divalent metals, including copper. It seems most probable that some replacement has indeed occurred in this case; from the copper estimation figures however, not more than one in every ten chlorophyll molecules has been affected.

In view of the influence apparently exercised by magnesium on the reactivity of the chlorophyll molecule (106), some doubt existed as to the effect of this//

this partial substitution on the photo-oxidation. Furthermore, in the dyeing industry, the photo-activity of many dyes is frequently diminished by treating the fibre with copper acetate (105). In recent years, Okuntsov (107) has reported the use of copper sprays in delaying the decomposition of chlorophyll in living plants.

Therefore, although copper substitution to the very slight extent involved would be expected to have an inappreciable effect on the oxygen: pigment molecular ratio, it was considered desirable to obtain other preparations of chlorophyll and study their photo-oxidation under similar conditions.

The first of these was supplied by the same firm, but, as will be shown later, its purity proved to be of a low order. To obtain a suitable standard for comparison it was necessary finally to extract chlorophyll directly from spinach leaves and carry out its purification in the laboratory under carefully controlled conditions.

The investigation of these two preparations will be presented in turn below.

Examination of the second chlorophyll preparation. ("Copper-free").

The solid paste was extracted and purified on a bonemeal column, as described previously. The oxidation of the purified product was examined in Osira "white light" and at 100 m.m. pressure of oxygen, as follows:-

(10) Normal Oxidation//

(10) Normal Oxidation.

Conditions.

(a) 0.3 grams thallous bromide.

3.35 ml. purified chlorophyll solution 0.938 g/l. in acetone.

Vessel + gauge volume = 85.2 ml.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.6	1.20	10.5	0.46	25.8	0.24	31.8	0.25
1.8	1.10	11.6	0.40	26.9	0.22	33.2	0.23
2.6	1.00	12.9	0.38	27.4	0.72	33.8	0.56
4.1	0.92	13.7	0.37	27.7	0.54	34.2	0.37
5.8	0.78	16.0	0.35	28.2	0.36	35.0	0.28
7.2	0.68	17.8	0.33	28.6	0.34	35.9	0.26
8.5	0.60	21.8	0.29	29.9	0.27	37.0	0.24
9.6	0.50	24.5	0.26	31.0	0.26	38.3	0.23
						40.7	0.21

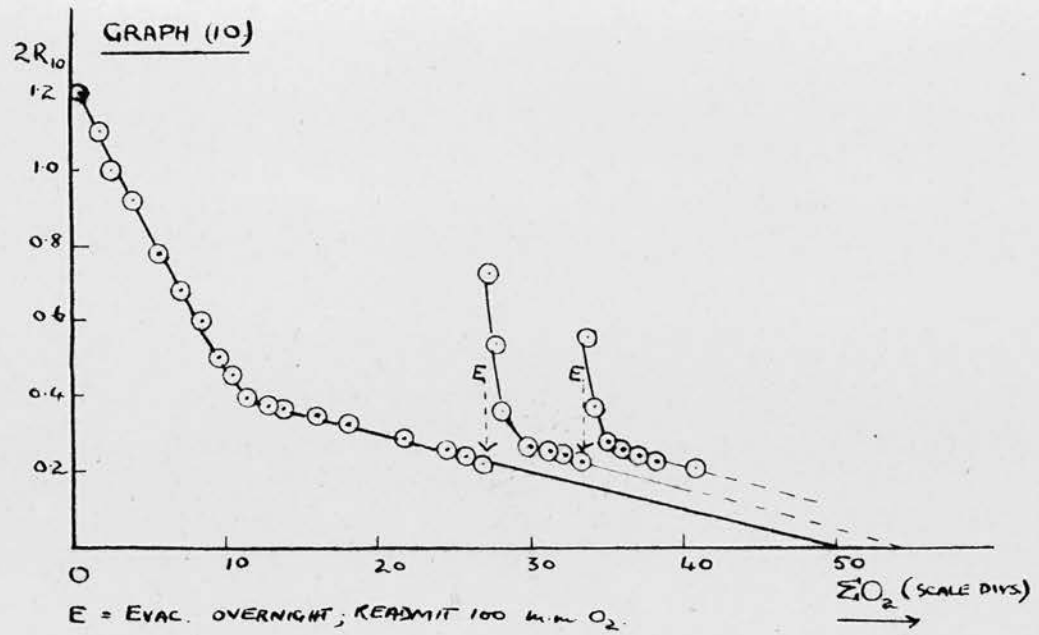
Total ΣO_2 (extrapolated value) = 50 scale divisions.

$\therefore \frac{\text{oxygen}}{\text{chlorophyll}}$ = 2.04 molecularly.

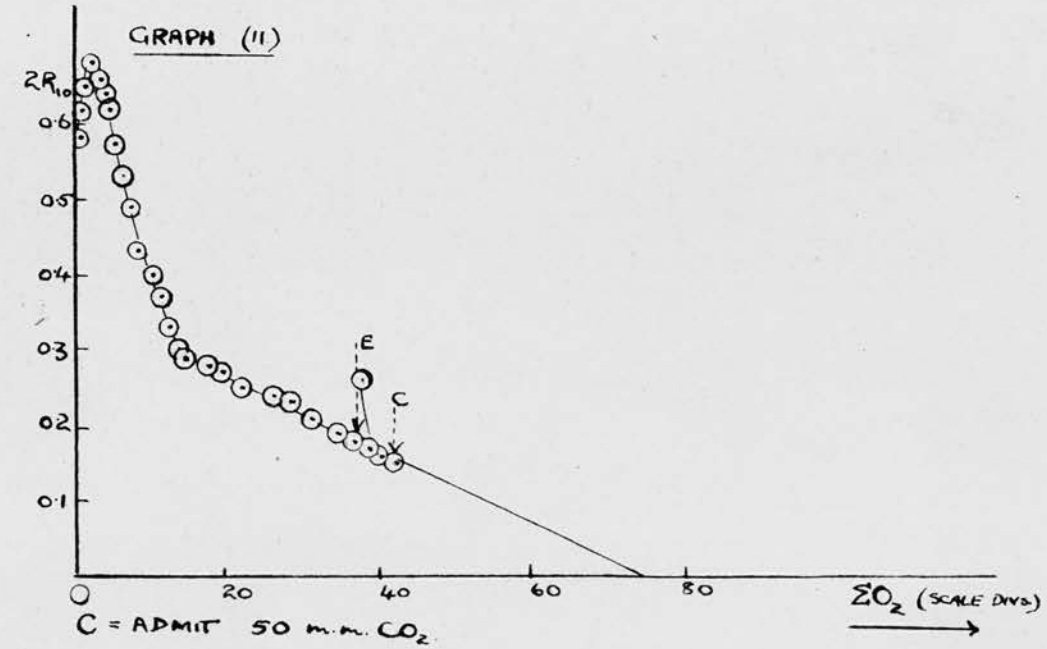
(b) The experiment was repeated to test the effect of water vapour, carbon dioxide and hydrogen. As before, no effect could be detected in any instance.

It is obvious from the above result that this copper-free sample takes up twice the amount of oxygen per molecule of pigment as was absorbed by the first preparation. The reaction is again reversible, although to a much smaller extent, and the shape of the reaction curve is quite different from that of purified "copper-chlorophyll", consisting of 2 sections, AB and BC.

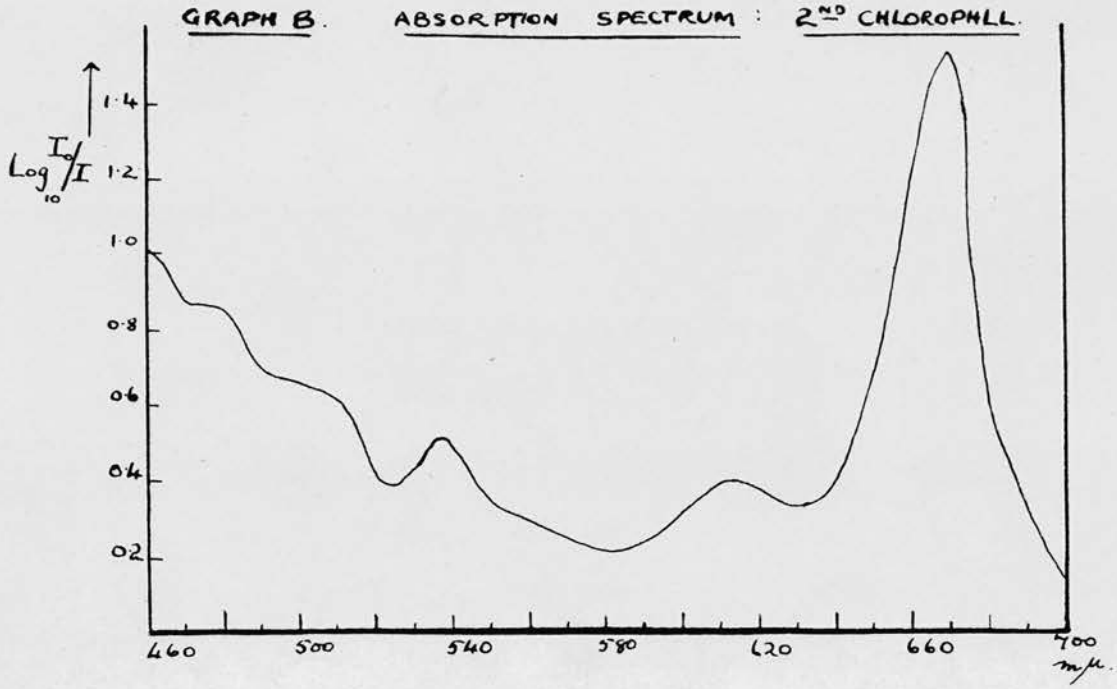
This//



E = EVAC. OVERNIGHT; READMIT 100 m.m O_2.



C = ADMIT 50 m.m. CO_2.



This second sample was found to differ from the first preparation in certain other respects listed below:-

(1) The second sample gave the phase test and showed a bright red fluorescence in ultra-violet light.

(2) The colour of its solution was a decided olive-green, in contrast to the emerald green of the copper-containing sample.

Its absorption spectrum was therefore determined in acetone, and certain distinct differences from the first sample, especially the position of the red maximum, were noted.

Acetone solution of second chlorophyll preparation (bonemeal-purified).

Results. GRAPH B.

λ	$\text{Log}_{10} I_0/I$	λ	$\text{Log}_{10} I_0/I$	λ	$\text{Log}_{10} I_0/I$
700	0.14	610	0.41	525	0.40.
690	0.30	600	0.32	520	0.40
680	0.62	590	0.27	510	0.63
670	1.54	580	0.22	500	0.86
665	1.46	570	0.25	490	0.70
660	1.20	560	0.30	480	0.87
650	0.67	550	0.34	470	0.87
640	0.40	540	0.50	460	1.01
630	0.33	535	0.52	450	1.80
620	0.39	530	0.45		

Concentration = 0.172 g/litre; l = 1.000 cms.

The spectrum was not changed significantly by using other solvents, such as ether or alcohol.

It was believed at the time that all the points detailed above could be related to the effect of copper on the molecule.

Effect//

Effect of Acids:

(11) Conditions:

0.3 grams thallous bromide.

3.35 ml. purified chlorophyll 0.938 g./l. in acetone.

0.96 ml. phosphoric acid 0.322 g./l. in water.

Volume of vessel + gauge = 85.2 ml.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.3	0.58	6.1	0.53	17.2	0.28	36.4	0.18
0.9	0.62	7.2	0.49	19.8	0.27	38.0	0.26
1.5	0.65	8.1	0.43	20.8	0.26	38.6	0.17
2.2	0.68	10.3	0.40	22.0	0.25	39.6	0.16
3.2	0.66	11.4	0.37	26.3	0.24	41.5	0.15
3.8	0.64	12.4	0.33	28.3	0.23		
4.5	0.62	13.9	0.30	31.1	0.21		
5.1	0.57	14.6	0.29	34.5	0.19		

Total ΣO_2 = 75.0 scale divisions.

$\therefore \frac{\text{oxygen}}{\text{chlorophyll}}$ = 3.05 molecularly.

(12) Conditions.

0.3 grams thallous bromide.

3.32 ml. purified chlorophyll 0.938 g./litre. = 3.45 μmols

1.13 ml. pyruvic acid 0.269 g./l. in water. 3.5 μmols

Volume of vessel gauge = 86.9 ml.

MR 2.99

Results.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.2	0.44	6.3	0.38	16.7	0.20
1.4	0.52	7.0	0.36	19.7	0.19
2.4	0.50	8.9	0.30	22.1	0.18
3.4	0.48	11.1	0.27	27.2	0.16
4.7	0.43	13.6	0.22	34.0	0.15
5.6	0.40	14.4	0.21	35.8	0.12

Total ΣO_2 = 71.4 scale divisions.

$\therefore \frac{\text{Oxygen}}{\text{chlorophyll}}$ = 2 .99 molecularly.

The graph obtained from these results has been omitted, since it was similar in all essential features to that of Experiment (11).

It had been expected that the addition of acid would have the same effect on the second preparation. as on the copper-substituted sample, namely the formation of pheophytin with the production of two new centres of oxidation, giving 4:1 molecular ratio for the second sample. However, the value remained at 3:1, just as for the original preparation.

There were then two possibilities:-

(a) This mechanism did not hold for the second sample.
(b) There was already some pheophytin in the second preparation.

The second possibility was indeed shown to be the case, for the following reasons:-

(1) The absorption spectrum of the second preparation was compared with that reported by Zscheile for pure//

pure chlorophyll and for pheophytin/chlorophyll mixtures (18). The maximum at 6700 A, and the occurrence of the characteristic band in the region of 5300 - 5350 A were taken as definite evidence of the presence of pheophytin in the second preparation.

(2) Pure pheophytin was prepared and found to have an oxygen uptake ratio of 3:1 molecularly.
(See Experiment (13) ahead).

(3) Pure chlorophyll was prepared by extraction from spinach leaves and found to take up one molecule of oxygen per molecule of pigment, just as the original copper-substituted sample. (see Experiment (14)).

The oxygen: pigment molecular ratio of approximately 2:1 for the second preparation could be explained by the presence of 50% pheophytin. This substance is a common impurity in preparations of chlorophyll; according to Zscheile, it is unavoidable when a solid product is formed. Because of its marked similarity to chlorophyll, their separation is almost impossible.(18)

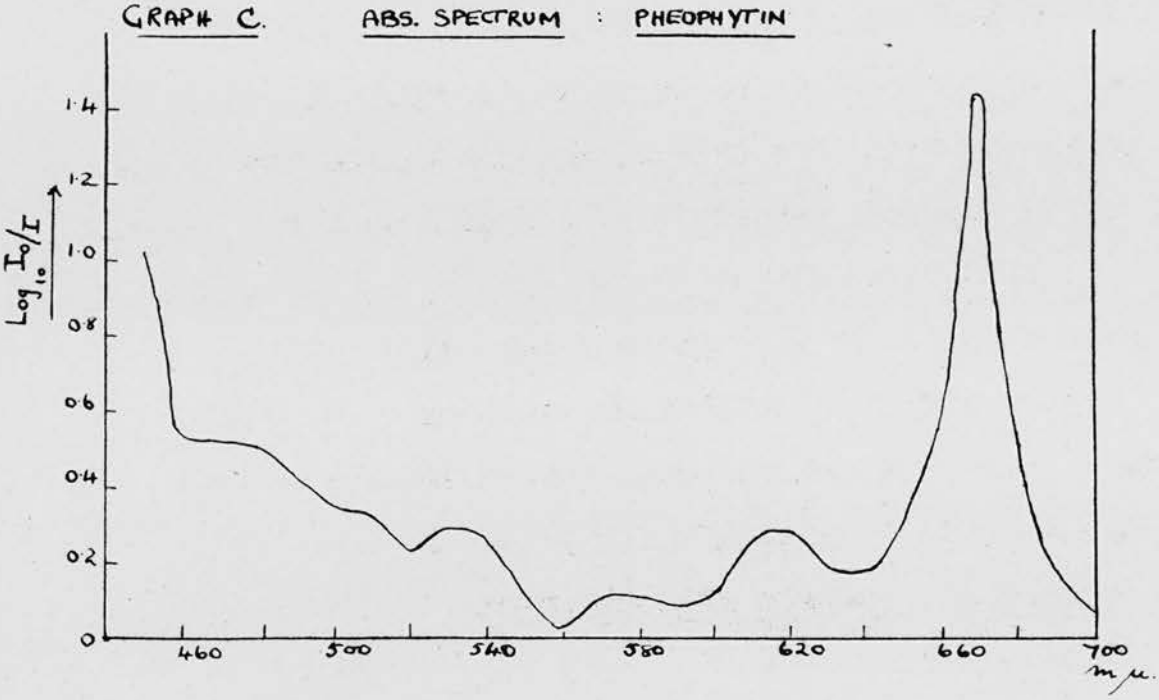
Preparation of pure pheophytin.

The method described by Willstatter (108) for the preparation of pheophytin from chlorophyll in grass was adapted for work on a much smaller scale, as follows:-

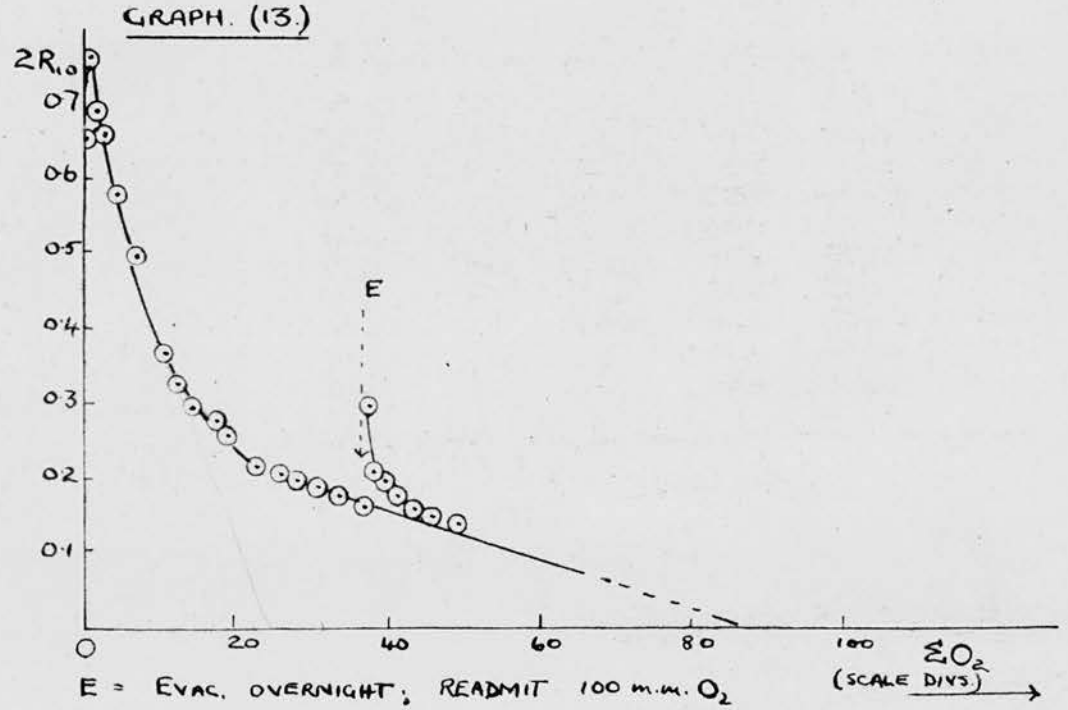
25 mls. of the solution of the bonemeal-purified second preparation (0.938 g./litre in acetone) were shaken overnight with 0.02 grams oxalic acid crystals.

The//

GRAPH C. ABS. SPECTRUM : PHEOPHYTIN



GRAPH. (13.)



E = EVAC. OVERNIGHT; READMIT 100 m.m. O_2

The resulting solution was extracted with a chloroform/water mixture and then evaporated to dryness. The film obtained was extracted with water until the extractions gave no reaction on heating with acid permanganate. The film of pheophytin was then dried over phosphorus pentoxide to constant weight. It was dissolved in a measured volume of acetone to give a concentration of 1.07 grams per litre.

This solution still fluoresced and gave the phase test, these reactions being characteristic of both chlorophyll and pheophytin (13 and 108).

Absorption Spectrum of pheophytin.

GRAPH C.

λ (mμ)	$\text{Log}_{10} I/I$	λ (mμ)	$\text{Log}_{10} I/I$	λ (mμ)	$\text{Log}_{10} I/I$
700	0.07	620	0.29	530	0.30
690	0.18	610	0.29	525	0.25
680	0.47	600	0.12	520	0.23
675	0.84	590	0.08	510	0.33
670	1.44	580	0.12	500	0.36
665	0.92	570	0.13	490	0.43
660	0.63	560	0.03	480	0.52
650	0.36	550	0.12	470	0.54
640	0.18	540	0.27	460	0.54
630	0.18	535	0.30	450	1.03

l = 1.000 cms.

c = 0.134 g./litre in acetone.

(13) Oxidation of pheophytin.

Conditions:

0.3 grams thallous bromide.

3.32 ml. pheophytin solution 1.07 gram/litre in acetone.

Vessel + gauge volume = 85.2 ml.

Results//

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.2	0.64	10.3	0.37	25.7	0.21	37.9	0.30
0.8	0.75	12.6	0.33	28.1	0.20	38.6	0.21
1.9	0.69	14.6	0.30	30.7	0.19	39.5	0.20
2.9	0.66	17.5	0.28	33.6	0.18	41.6	0.18
4.6	0.58	19.3	0.25	37.0	0.16	43.3	0.16
7.0	0.50	22.6	0.22	37.4	0.46	46.0	0.15
						49.0	0.14

Total ΣO_2 (extrapolated) = 85.7 scale divisions.

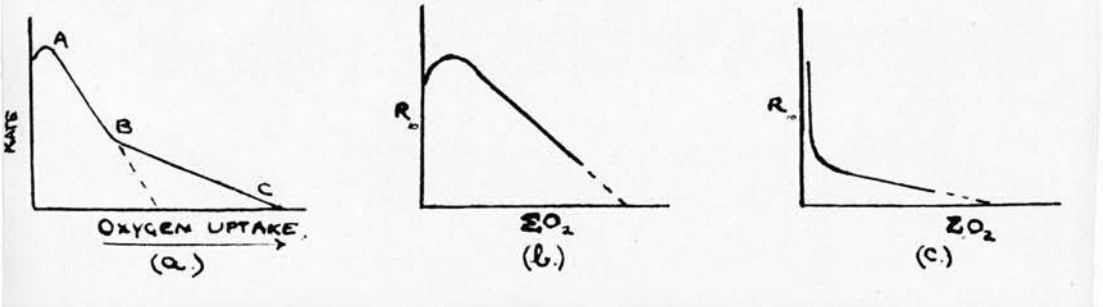
The molecular weight of a mixture of pheophytin a and b was taken as 879.7

Therefore, $\frac{\text{oxygen}}{\text{pheophytin}}$ = 3.00 molecularly.

Comparison of Reaction Curves.

This is perhaps a convenient stage to compare some of the recently detailed reaction curves relating to pheophytin.

As a further proof of the existence of pheophytin in the second "chlorophyll preparation" we have the decided similarity between the shape of its reaction curve and that of pure pheophytin, both being of the type (a), i.e. composed of two almost distinct sections:-



This is in marked contrast to the curve obtained with pure chlorophyll which has the form (b). This difference in shape may be of some significance, especially as in the case of pure pheophytin at least, the first section AB gives an extrapolated value of approximately one third the total oxygen uptake, which would correspond to the oxidation of one of the three active positions in the molecule.

Indeed, the type (a) curve could be considered as the sum of two curves, one of type (b) and the other of shape (c) which is strictly linear only in the final stages. These proposed 'component curves' could represent the concurrent oxidations of pheophytin in case (b) at the normal C_{10} position and in (c), at two of the central nitrogen atoms. The analysis of curve (a) in this manner, while apparently justifiable empirically, implies that the photo-oxidations of pheophytin at positions (b) and (c) can be regarded as independent. In a resonating structure such as pheophytin, this is not strictly true, and the oxidation at one position of the molecule would normally be expected to affect the other reactive centres. However, since C_{10} does not come within the routing of the all-round conjugated ring, its reactions may be to some extent independent.

The pigment in experiments (5) (11) and (13) is the same, namely pheophytin, whether formed before or after deposition on the thallous bromide, the only difference//

difference being the presence of acid actually in the film in the case of the first two experiments. With every acid-treated product, the molecular ratio of oxygen to pigment was found to be 3:1, and excluding for the moment those films which also contained carotene (Experiments (4), (7) and (8) and consequently showed an intial constant rate period, the shape of the reaction curves obtained was consistently of the type (a).

It was observed that on evacuating such pheophytin films and then continuing the oxidation under exactly the same conditions as before, a marked increase in the rate of pressure decrease occurred. This effect was of very short duration, since the rates without exception fell rapidly again to join the original curve. (In certain cases, the points obtained at this stage were very slightly above the curve, but at the slow rates involved, their position was never appreciably outwith the error of measurement.)

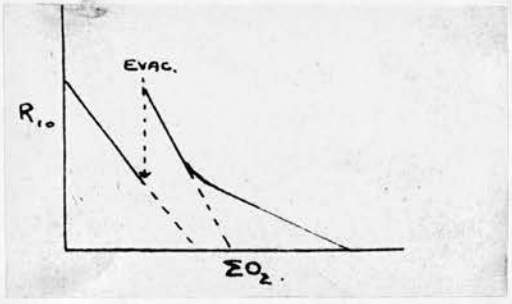
This temporary increase in reaction rate occurred both in the presence and in the absence of acid, and also with films to which acetone had been added <u>after</u> deposition of the acid - to improve the distribution of the pigment. It cannot therefore be explained by the rupture of any "protective acid layer" on evacuation.

Since no appreciable change in the extrapolated value for the total oxygen uptake occurred after evacuation//

evacuation, the photo-oxide of pheophytin was assumed to be irreversible. Each molecule of this pigment takes up three molecules of oxygen involving, according to the theory already proposed, three reactive centres - two being the positions where magnesium has been detached and the other being the "normal" oxidation position of chlorophyll, C_{10}. The oxide at this latter position is reversible in intact chlorophyll i.e. before its conversion to pheophytin, and these temporarily increased rates with pheophytin could be explained by assuming that, in this case also, the C_{10} oxide is reversible but only to a very slight extent. In pheophytin, due to the removal of the co-ordinately bound magnesium atom, the molecule has decreased resonance possibilities, and therefore one could expect that any intermediate dissociable oxide formed at C_{10} would be much less stable than that of chlorophyll. Consequently, it would tend to change into the stable non-dissociable form very rapidly. In this way, one could explain why evacuation never produced sufficient reversion to show a significant change in the oxygen uptake, although the rates were increased for a short period, extending for 2 to 3 scale divisions. Such slight reversion, of the initial curve AB, would pass unrecognised in the extrapolated gas uptake value.

Furthermore, with such "acid films" evacuation was generally not applied until considerable oxidation had//

had occurred. At this stage, very little of the C_{10} oxide could be expected to exist still in the reversible form. In one instance however, evacuation was deliberately applied at an early stage in the oxidation, while the rates still lay on the section AB; it was found that definite reversion had occurred, the graph being of the form:-



When pyruvic acid was used to prepare pheophytin and was present in the film, no increase in rates whatever was obtained on evacuation. Probably in this case, the acid concerned formed some stable complex with the C_{10} peroxide, preventing even a slight reversion.

Examination of the third chlorophyll preparation.

The last chlorophyll sample was obtained from spinach leaves by following one of the most recent extraction methods, already described in detail in an earlier section. The absorption spectrum of the mixed pigments, chlorophylls a and b, after removal of carotenoids by a bonemeal separation, agreed satisfactorily with the data published by Zscheile (18) and by Rabinowitch (24). In this case, there was no evidence from the curve of any degradation products//

products, such as pheophytin.

Absorption Results.

Graph D.

λ mμ	$\text{Log}_{10} I_0/I$	λ	$\text{Log}_{10} I_0/I$	λ	$\text{Log}_{10} I_0/I$
700	0.02	630	0.25	530	0.06
690	0.12	620	0.25	525	0.07
680	0.41	610	0.24	520	0.07
675	0.60	600	0.20	510	0.05
670	1.06	590	0.18	500	0.05
665	1.40	580	0.15	490	0.11
662	1.40	570	0.13	480	0.35
660	0.84	560	0.12	470	0.90
655	0.68	550	0.12	460	1.15
650	0.57	540	0.07	450	1.27
640	0.36	535	0.08	440	1.50

concentration = 0.144 g./l.

cell length = 1.000 centimetres.

Averaging several similar determinations, the maximum in the red end of the spectrum was found to lie between 6600 and 6620Å.

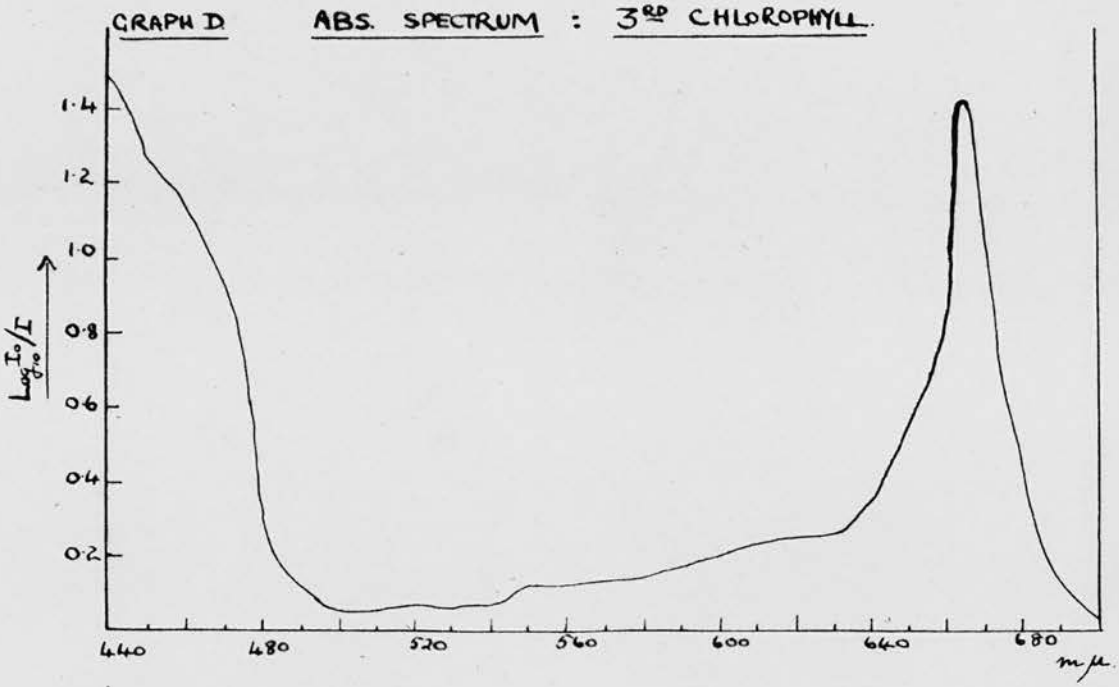
The absorption curve for an other solution of the same preparation showed no significant differences from these results.

In acetone solution, the preparation fluoresced bright red under ultra-violet light, and when dissolved in ether, it gave the "phase test" readily. As was expected, the preparation was much less stable than the copper-containing chlorophyll and if kept for any length of time in the solid state, its absorption spectrum indicated the formation of a small amount of pheophytin.

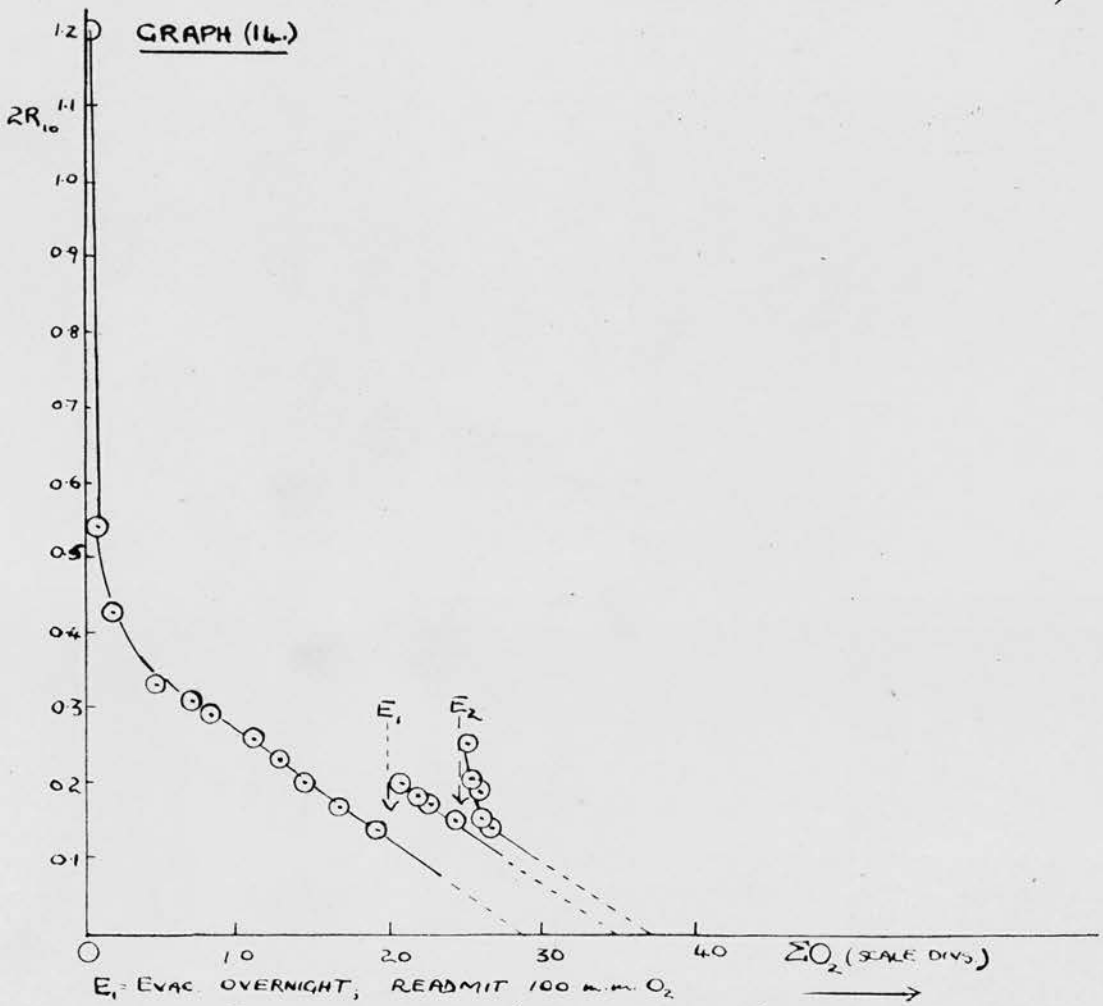
Care//

B

GRAPH D ABS. SPECTRUM : 3RD CHLOROPHYLL.



GRAPH (14.)



E_1 = EVAC. OVERNIGHT, READMIT 100 m.m. O_2

E_2 = " 30 MINUTES, ADMIT 1 m.m. H_2O, 20 m.m. CO_2 + 100 m.m. O_2

Care was therefore taken to store the preparation always in solution and in the dark.

The oxidation of the purified products, chlorophyll and carotenoids, were studied separately and also in mixtures, under the same conditions of oxygen pressure, illumination and temperature as before.

"Normal Oxidations":-

(14) chlorophyll.

Conditions:

0.3 grams thallous bromide.

3.32 mls. chlorophyll solution (bonemeal purified) 1.01 g./litre in acetone.

Vessel + gauge volume = 85.2 ml.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.4	1.2	8.4	0.29	19.2	0.14	25.0	0.25
0.9	0.54	11.2	0.26	20.6	0.20	25.4	0.20
1.8	0.43	12.8	0.23	21.7	0.18	25.6	0.19
4.8	0.33	14.5	0.20	22.4	0.17	25.9	0.15
7.1	0.31	16.7	0.17	24.1	0.15	26.5	0.14

Total ΣO_2 i.e. extrapolated value = 28.6 scale divisions.

∴ oxygen/chlorophyll = 1.09 molecularly.

The film, on removal, appeared considerably bleached. This was confirmed by taking the absorption spectrum of an acetone solution of the product, a marked depression of the red maximum (to 30% of the original value) being very obvious.

(15) Carotenoid//

(15) Carotenoid Fraction.

Conditions:

0.3 grams thallous bromide.

2.85 mls. of carotenoid solution 0.64 g./litre in acetone.

Vessel + gauge volume = 85.2 mls.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.1	0.19	9.0	0.18	12.2	0.14
4.0	0.19	10.2	0.17	13.0	0.13
8.3	0.19	11.4	0.15	14.5	0.11

The graph obtained was similar in shape to those obtained with the carotenoid fraction from the original commercial preparation. The constant rate period was again observed, and the reactio n was slightly reversible. Rates were however much slower than those for the original preparation, and this might indicate the presence of some anti-oxidant (109)

Total ΣO_2 (extrapolated value) = 23.5 scale divisions.

∴ Oxygen/carotenoid = 1.04 molecularly.

(16) Mixture.

Conditions:

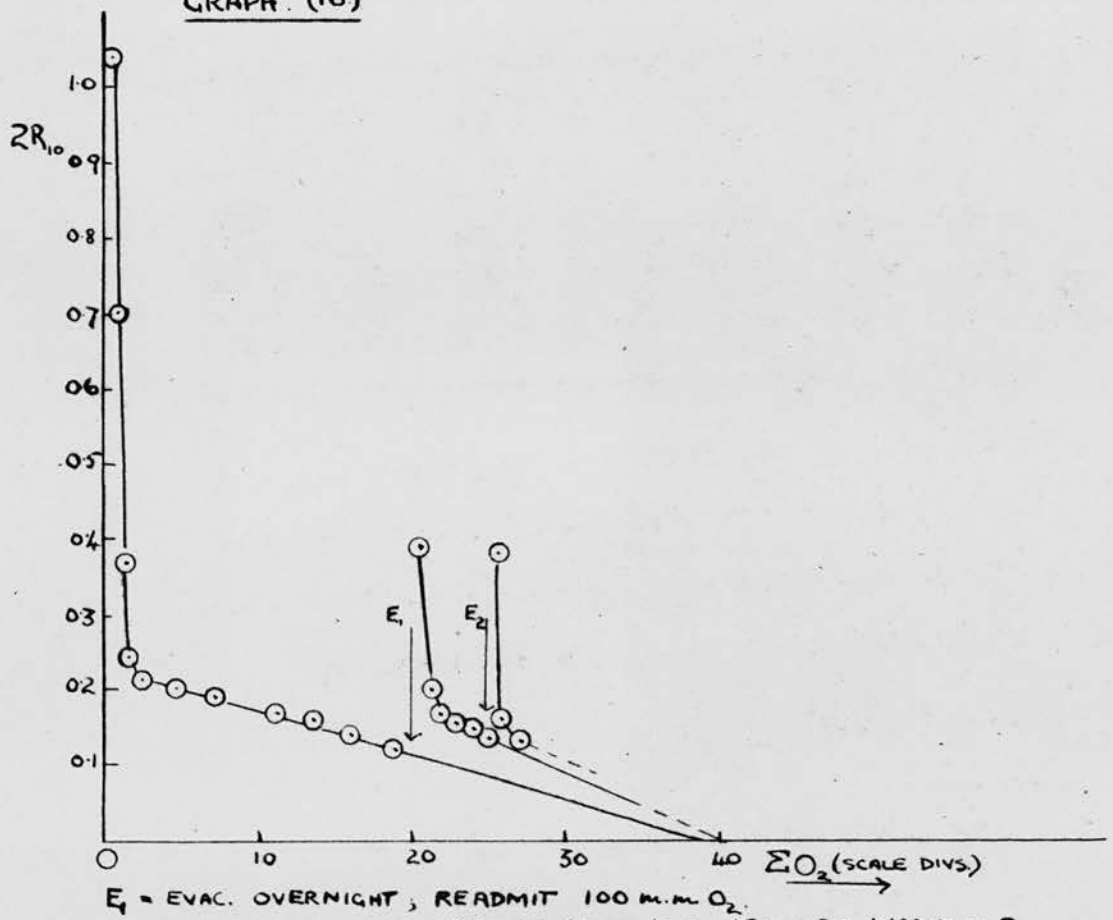
0.3 grams thallous bromide.

2.85 mls. chlorophyll solution, 1.01 g./litre in acetone.

1.89 mls. carotenoid solution, 0.64 g./litre in acetone.

Volume of vessel + gauge = 86.9 ml.

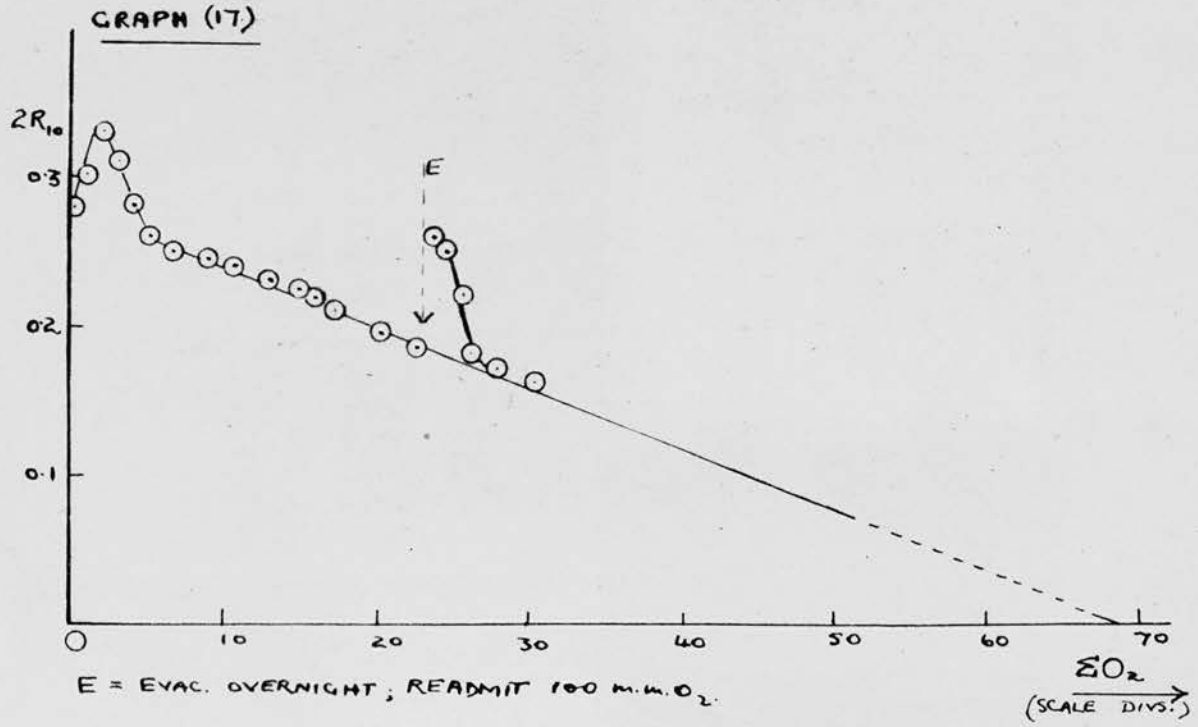
GRAPH. (16)



E_1 = EVAC. OVERNIGHT; READMIT 100 m.m O_2.

E_2 = " 10 MINUTES; ADMIT 1 m.m. H_2O, 10 m.m. CO_2 + 100 m.m. O_2.

GRAPH (17)



E = EVAC. OVERNIGHT; READMIT 100 m.m O_2.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.5	1.04	4.8	0.20	18.9	0.12	24.8	0.14
1.0	0.70	7.2	0.18	20.3	0.39	25.4	0.38
1.5	0.37	11.2	0.17	21.2	0.20	25.6	0.16
1.8	0.24	13.6	0.16	21.7	0.17	26.0	0.14
2.5	0.21	16.1	0.14	22.8	0.16	27.0	0.13

Total ΣO_2 = 38.2 scale divisions.

Assuming oxygen/carotenoid = 1:1, the oxygen uptake due to chlorophyll = 23.5 scale divisions.

∴ oxygen/chlorophyll = 1.06 molecularly.

(17) Effect of acid.

Conditions:

0.3 grams thallous bromide.

2.85 mls. chlorophyll solution 1.01 g./litre in acetone.

1.04 ml. phosphoric acid 0.302 g./litre in water.

Volume of vessel + gauge = 86.9 ml.

Results.

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.3	0.28	5.2	0.26	14.9	0.225	23.7	0.26
1.1	0.30	6.9	0.25	15.6	0.22	24.5	0.25
2.1	0.33	9.0	0.245	17.1	0.21	25.6	0.22
3.0	0.31	10.5	0.24	20.1	0.195	26.2	0.18
4.1	0.28	12.9	0.23	22.5	0.185	27.8	0.17
						30.2	0.16

Total oxygen uptake = 67.7 scale divisions (extrapolated value)

∴ oxygen/chlorophyll = 3.07 molecularly.

The//

The absorption spectrum of the product showed a considerable depression of the red maximum, to 18% of its original value. It was now nearer 6700 Å than 6600Å; the appearance at 5000-5050Å and 5300-5350Å of the bands characteristic of pheophytin was also observed.

Summary and discussion.

(1) The oxygen: chlorophyll molecular ratio of unity confirms the result obtained with the first, copper-containing preparation and this "normal oxidation" was again reversible on evacuation. Furthermore, the rates of oxidation of these two preparations were of the same order.

(2) In the presence of acid, the oxygen uptake was once more increased threefold, and apart from the usual temporary increase in rates on evacuation, the reaction showed no obvious reversibility. The formation of pheophytin was confirmed by examining the absorption spectrum of the product.

(3) At the points shown on the graphs, carbon dioxide and water vapour were added in exactly the same manner as before. No significant effect on the oxidation was recorded in any instance.

(4) The shape of the "normal" reaction curve is slightly different from that obtained with the copper-chlorophyll. With this third preparation, a fairly rapid initial oxidation occurs, but this is followed by fairly slow rates, after only 15% of the total oxidation has occurred.

There//

There is evidence in certain cases of a slightly increased rate when the film has stood in oxygen in the dark overnight, indicating a slight reversal, even at 100 m.m. pressure.

(5) No straight line period was obtained with the film of mixed pigments (Experiment 16). When one considers the relative rates of oxidation however, it is obvious that the fast initial rates of chlorophyll oxidation would mask any constant rate period of the carotenoid component, the result being the reverse of that obtained with the pigments of the first preparation.

Effect of copper on the molecule.

These recent experiments showed quite clearly that in all essential features the oxidation of pure chlorophyll obtained from spinach leaves resembled that of the original copper-containing preparation. Both reactions, which proceed at rates of the same order, are reversible and have an oxygen uptake ratio of unity. Copper then has no obvious effect on the oxidation of chlorophyll, yet the two preparations differed in certain other respects which will now be discussed in more detail.

It has already been mentioned that the copper-containing chlorophyll did not fluoresce nor did it give the colour change known as the "phase test". Since this latter reaction is given by all intact chlorophyll//

chlorophyll preparations, it was suspected initially that this first sample was "allomerised" (22) i.e. probably already oxidised at one part of the molecule. However, by treating both the spinach chlorophyll and the second commercial preparation with a copper sulphate solution, a product was obtained which neither fluoresced nor gave the phase test and whose absorption spectrum, in both cases closely resembled that of the first preparation. The reaction could also take place in an atmosphere of nitrogen.

Experimental details for the copper treatment of the second commercial preparation.

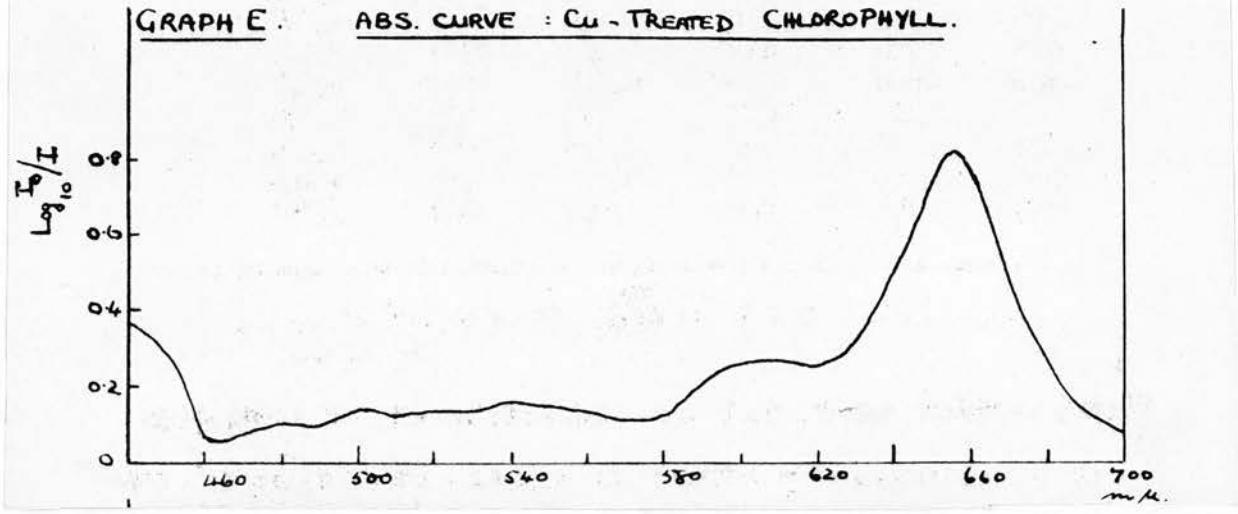
40 mls. of an acetone solution of bonemeal-purified "chlorophyll" (second preparation i.e. a chlorophyll/pheophytin mixture) of concentration 1.03 grams per litre was shaken for 24 hours with 5 mls. of an aqueous copper sulphate solution containing 0.1 gram copper per litre. The resulting mixture was extracted with ether and the extract evaporated under a filter-pump vacuum to constant weight.

The pigment was then dissolved in acetone to give a solution of suitable concentration. Its absorption spectrum, and finally its photo-oxidation on thallous bromide were examined exactly as before.

Absorption Spectrum:

GRAPH E//

GRAPH E. ABS. CURVE : Cu - TREATED CHLOROPHYLL.



GRAPH E.

λ mμ	$\text{Log}_{10} I_0/I$	λ	$\text{Log}_{10} I_0/I$	λ	$\text{Log}_{10} I_0/I$
700	0.08	620	0.24	530	0.13
690	0.13	610	0.26	520	0.13
680	0.26	600	0.26	510	0.12
670	0.50	590	0.22	500	0.13
665	0.64	580	0.11	490	0.09
660	0.78	570	0.11	480	0.11
655	0.82	560	0.13	470	0.07
650	0.76	550	0.15	460	0.06
640	0.51	540	0.16	450	0.26
630	0.34	535	0.15	440	0.37

l = 1.000 cms; c = 0.159 grams/litre in acetone.

The reaction curve for the oxidation of this copper-treated product was almost identical with that of the first chlorophyll preparation. The initial acceleration period occurred again, and is apparently associated with the presence of copper.

Summary.

It has been shown that the inability of a chlorophyll preparation to fluoresce and give the phase reaction, while attributable to various causes in different circumstances (22) can also be caused by the addition of copper.

From experience with the second and third chlorophyll preparations, it was obvious that copper-free samples could be degraded very easily to pheophytin, especially when stored in the solid state. This had already been observed by Zscheile who published a list of the milder conditions, such as drying, even at room temperature, which have proved sufficient to degrade//

degrade pigment preparations to varying extents.

The substitution of the centrally-bound magnesium by copper is a reaction which proceeds readily (105) and it seems most probable that this occurs in the formation of these "copper-chlorophyll" preparations. However, the copper content of the first preparation was 0.7%, which implies that approximately only one in every ten chlorophyll molecules has been copper substituted in this way.

From the forgoing considerations and experimental results, it appears that the function of copper as a "stabiliser" or colour preservative for plant extracts is associated not with preventing the oxidation of the pigment, but rather with delaying the formation of pheophytin in all except the more drastic (e.g. acid) conditions. The oxygen uptake values obtained after acid treatment indicate that both metals have been removed and the chlorophyll thereby converted completely to pheophytin.

Noack has reported (32) the formation of a similar compound, which he termed "copper-pheophytin". It was also non-fluorescent and capable of absorbing oxygen.

The "stabilising" action is no doubt connected with the fact that copper is a better co-ordinating metal than magnesium. But it is difficult to see how the presence of this small amount of copper affects the stability of the other chlorophyll molecules, at least//

least by any chemical means. The copper may of course have some physical effect on the unsubstituted molecules, by virtue of the magnetic field associated with the odd electron in its orbitals.

At any rate, we have a contrast here between this stabilising action of copper and its effect on various auto-oxidisable compounds, e.g. tetralin, where in certain forms, it acts as an auto-oxidation catalyst.

The foregoing experiments and discussion represent the investigation of the normal oxidation and of the oxidation in the presence of acid for each of the three chlorophyll preparations. One of the facts which the results have definitely established is that the copper-stabilised sample oxidises in an essentially similar fashion to the pure chlorophyll obtained from spinach leaves, the oxygen uptake in each case being identical.

Since the stabilised form could be kept intact for longer periods in solution, and was consequently much easier to handle, it was decided that, for the present, it should be used in preference to the more easily degraded "third preparation", in any further experiments.

These additional experiments will now be described in turn. It is obvious that in dealing with a substance such as chlorophyll, so closely connected with auto-oxidations generally and also with the reactions of//

of photosynthesis, there is an almost unlimited number of ways in which this investigation could be extended and the reaction examined under widely different conditions and from various angles.

It has already been mentioned that, in its oxidation kinetics, chlorophyll resembles many other organic molecules which, from simple hydrocarbons such as tetralin to the more complex structures such as rubbers, all follow the same general scheme. It has ofter been reported that such auto-oxidations in the liquid phase can be catalysed by certain metal salts, the most active of these being cobalt naphthenate (110). The metal ion in these catalysts is capable of undergoing a valency change and is believed to act by catalysing both the formation and the decomposition of the peroxides involved in the reaction.

It was therefore decided to study the oxidation of chlorophyll in the presence of certain cobalt salts, including cobalt naphthenate.

Preparation of Cobalt Salt Solutions.

(a) Cobalt Naphthenate.

The cobalt naphthenate used was a commercial preparation obtained from Hopkins, Williams & Co. It was insoluble in water and only sparingly soluble in most of the common organic solvents. The most suitable was found to be ether, although a small insoluble residue remained in this case also.

The//

The solid was therefore extracted with ether and the solution decanted from the residue. Its concentration was determined by evaporating measured portions in crystallising dishes under a filter pump vacuum. An analysis of a sample of the solid by Weiler and Strauss gave the percentage cobalt as 15% by weight.

(b) Oxycobaltiac Ammonium nitrate.

This was prepared according to Werner's method (6). The black crystals obtained were dried over phosphorus pentoxide in a vacuum dessicator. It was insoluble in most organic solvents, but very soluble in water. The solution was unstable in that after some time, solid settled out, giving a fine suspension. It had therefore to be constantly renewed. Concentrations were determined by dissolving weighed amounts in measured volumes of water.

Formula: $(Co(NH_3)_5 \cdot O_2 \cdot (NH_3)_5 Co) (NO_3)_4$

(c) Disalicylalethylenediimine cobalt.

The preparation and purification of this compound were carried out according to the method described by Diehl (5). It was quite insoluble in water, but it was possible to prepare solutions in benzene and acetone.

Cobalt Naphthenate Experiments.

Films were prepared in the same way as before, by depositing the chlorophyll on the thallous bromide, adding the measured amount of salt solution and evaporating//

evaporating the solvent once more. In most experiments, the amount of solution used was such as to give a salt/chlorophyll ratio of approximately 1:10 by weight.

The cobalt salt solution was found to deteriorate slowly (cf.110), since storing for any length of time led to a decrease in the efficiency of the catalyst. It had therefore to be renewed every week.

To test whether the salt itself took up oxygen under the conditions of the experiment, a "blank" experiment was first carried out as follows:-
5 mls. of the cobalt naphthenate solution (0.06 g/litre in ether) were added directly to 0.3 grams thallous bromide; the solvent was then evaporated, and the film evacuated in the usual way.

It stood for several hours:-

(a) In vacuo in the dark and then illuminated.

(b) In 100 m.m. of oxygen in the dark and then illuminated.

No significant pressure change was observed in either case.

Oxidation of the chlorophyll/carotenoid mixture in presence of cobalt naphthenate.

Conditions:

0.3 gram thallous bromide.

3.35 ml. chlorophyll/carotenoid mixture 1.144 g/l. in acetone.
5.0 ml. cobalt naphthenate solution in ether 0.06 g./litre.

Vessel + gauge volume = 86.9 ml.

Results//

Results.

(18).

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.7	0.21	10.0	1.50	27.6	0.86	42.3	0.20
1.5	0.42	16.4	1.50	29.2	0.75	43.5	0.16
2.6	0.62	19.2	1.28	30.6	0.70	45.4	0.12
4.0	0.90	21.5	1.12	32.4	0.58	45.6	0.30
6.0	1.26	23.6	1.05	35.0	0.49	48.0	0.30
7.6	1.50	25.7	0.94	36.0	0.42	51.7	0.24

Total ΣO_2 (extrapolated) = 46.5 scale divisions for the dark reaction.

Summary.

(1) After an acceleration period of approximately one hour, the pressure decreased at a considerable rate without any illumination. This effect was rather unexpected and it was therefore confirmed on several films. In the absence of oxygen, no pressure change whatever was observed - even on illumination.

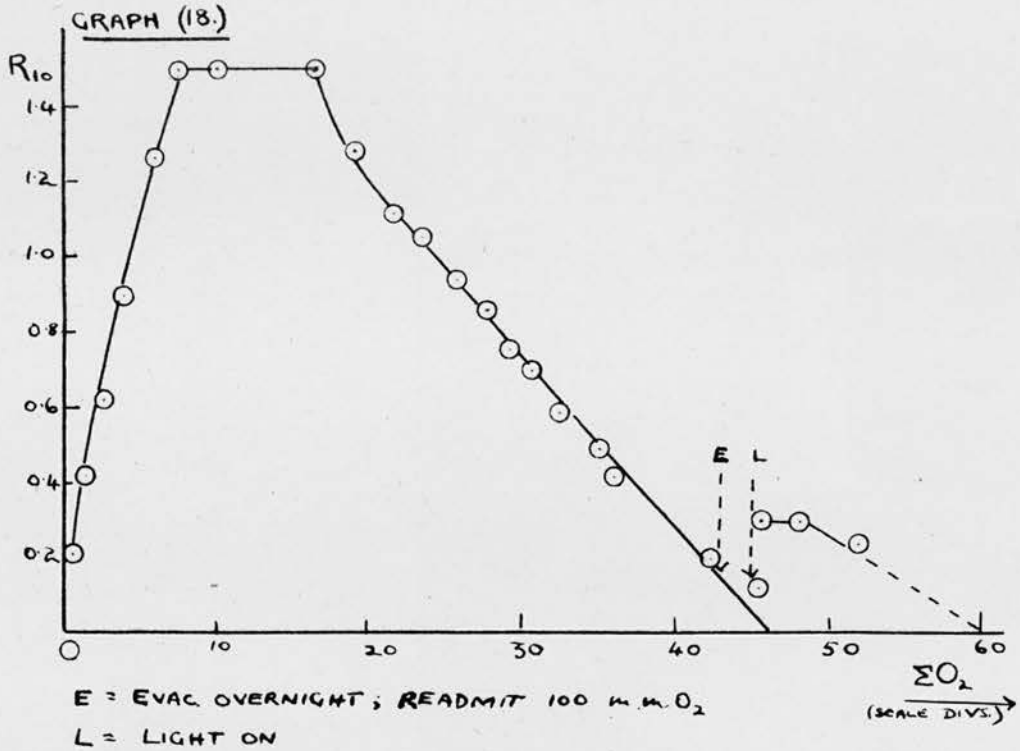
(2) The rates obtained were at least three times faster than those of the previous experiments, and were increased slightly by illumination with the Osira light.

(3) The general shape of the reaction curve resembled previous results - two differences being, in this case, an extended acceleration period and a longer constant rate period.

(4) This reaction, unlike the normal oxidation, could not be reversed by evacuating and then re-admitting oxygen.

(5) Examination//

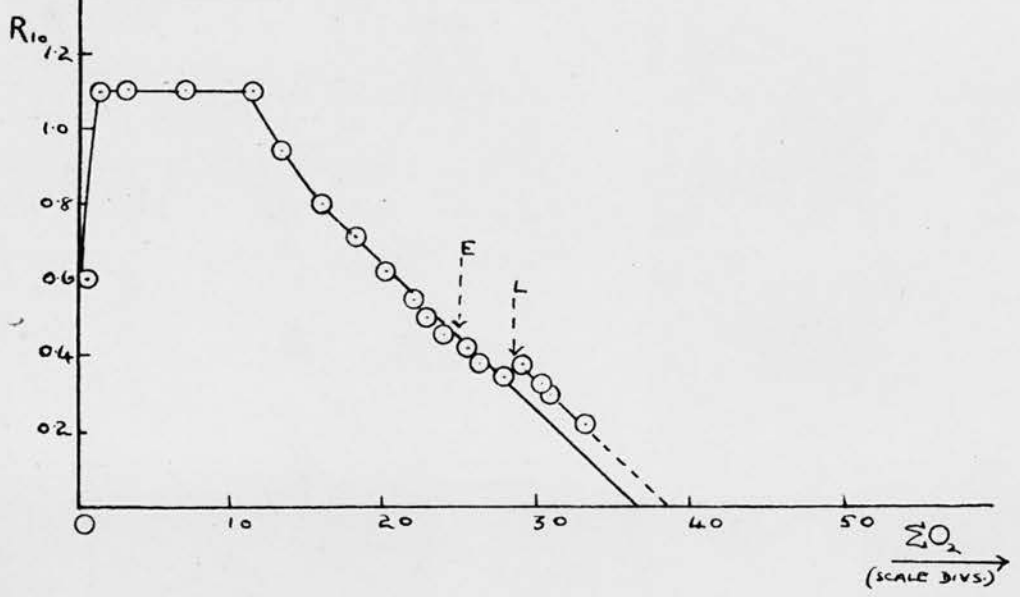
GRAPH (18.)



E = EVAC. OVERNIGHT; READMIT 100 m.m. O_2

L = LIGHT ON

GRAPH (20)



(5) Examination of the reaction products.

(a) After the completion of the experiment, the chlorophyll film appeared very bleached. Its absorption spectrum in acetone solution showed a pronounced depression of the red maximum, indicating that the chlorophyll component had definitely been oxidised.

(b) A second similar film, subjected to the same conditions was extracted with water and the extract tested for peroxide reaction (92). The test, however proved negative.

(6). The oxygen uptake value for this dark reaction was significantly and consistently greater than that obtained in the absence of cobalt naphthenate. The uptake was increased slightly if the film was illuminated.

The effect of the salt on the two pigments separately was next examined.

(19) Oxidation of pure chlorophyll in the presence of cobalt naphthenate.

0.3 gram thallous bromide.

3.35 mls. chlorophyll solution (from bonemeal separation) 1.0 g./litre in acetone.

5.0 ml. cobalt naphthenate solution 0.06 g./l. in ether.

Volume of vessel + gauge = 86.9 ml.

Results.

No dark uptake was observed with this film, and only the ordinary slow rates on illumination. The experiment was therefore repeated using a fresh solution//

solution of the salt. The effect was exactly the same, i.e. no dark reaction in the absence of carotenoids. In this case the reversibility on evacuation was examined. It was found that just as in the normal oxidation of the pigment, a reversible oxide was formed. In the same manner as before, water vapour and carbon dioxide were admitted to the system. Again, however, no significant effect was observed.

Apparently, from the results of these experiments, the oxidation of chlorophyll is unaffected by cobalt naphthenate when carotenoids are absent from the film.

(20) Oxidation of the carotenoid fraction in presence of cobalt naphthenate.

Conditions:

0.3 grams thallous bromide.

3.35 ml. carotenoid solution 0.90 g/litre in acetone.

3.5 ml. cobalt naphthenate solution in ether, 0.10 g./litre.

Vessel + gauge volume = 85.2 ml.

Results.

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.6	0.6	13.4	0.94	22.9	0.50	29.0	0.37
1.5	1.10	16.0	0.80	23.9	0.45	30.2	0.32
3.0	1.10	18.3	0.71	25.4	0.42	30.7	0.30
7.0	1.10	20.2	0.62	26.3	0.38	33.1	0.22
11.4	1.10	22.0	0.55	27.8	0.34		

Total ΣO_2 (extrapolated) = (36.6 scale divisions in the dark.
(41.5 scale divisions on illumination.

Summary//

Summary.

(1) As for the mixed pigments, a reaction proceeded in the dark, but the rates were not so fast as those of Experiment (18). Again, the reaction was irreversible on evacuation.

(2) As before, the rates and the oxygen uptake were increased on illumination.

(3) The duration of the acceleration period was considerably reduced with the carotenoid film. There was therefore an increased possibility in this case that some oxidation occurred during the preparation of the film. Because of this, the experiment was repeated several times to confirm the oxygen/carotenoid molecular ratio for the dark uptake. The values obtained were on the average close to unity, being 1.02, 0.98 and 0.97 but with one film, which showed a longer acceleration period, a value of 1.40 was actually obtained.

It was found that increasing the amount of cobalt naphthenate in these experiments did not produce any increase in rate or in oxygen uptake. It was therefore obvious that carotene was not sensitising any oxidation of the naphthenate, while the result of experiment (19) dismissed this possibility for chlorophyll also. In every experiment, the total oxygen uptake obtained definitely depended on the amount of pigment present. To test whether the thallous bromide present in these films was participating in any//

any way in the reaction, a film was prepared on talc, as follows:-

(2 Oxidation on talc.

0.1 gram talc.

3.35 ml. chlorophyll-carotenoid mixture 1.126 g./litre in acetone.
2.5 ml. cobalt naphthenate solution 0.13 g./litre in ether.

Volume of vessel + gauge = 85.2 ml.

Results.

ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.8	0.23	16.6	0.16	light on:-	
2.3	0.27	17.5	0.13	22.0	0.16
5.0	0.27	19.6	0.12	25.0	0.14
10.0	0.27	21.0	0.11		
15.7	0.27				

Total ΣO_2 (extrapolated) = 45.0 scale divisions for the dark reaction.

The graph for this reaction is essentially similar to that of Experiment (18)

Summary.

A dark reaction was again obtained, with an oxygen uptake value of the same order as before. The rates could be accelerated by illumination, but the maximum dark rate was much less than on thallous bromide. This however could be due to deterioration of the catalyst, and it appears quite definite that the reaction is essentially the same whether talc or thallous bromide is used as adsorbent.

Increase in the oxygen uptake with cobalt naphthenate films.

The//

The oxygen uptake values obtained with the films of mixed pigments showed that much more oxygen was being absorbed than could be accounted for by the normal oxygen/pigment molecular ratio of 1:1. The experiments with the carotenoid fraction indicated that on the average, the oxygen uptake ratio of 1:1 was unchanged for this pigment. However, these reactions do not require light initiation, and although readings were taken as soon as oxygen had been admitted to the system and the films were always prepared in the least possible time, the oxygen uptake value obtained must, because of the possibility of oxidation during the preparation of the film, be a minimum figure. This is especially true in the case of the carotenoid films, since their induction or acceleration period is much shorter. This is borne out by the fact that as already mentioned, with one exceptional film of carotenoid on thallous bromide, a molecular ratio of 1.4:1 was obtained, the reaction in question showing a much longer induction period than usual. These experiments were repeated with further cobalt salts, these being:-

(1) oxycobaltiac ammonium nitrate.

(2) disalicylalethylenediimine cobalt.

(3) cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$.

The first two salts were used because they had been quoted (6) as capable of undergoing reversible oxygenation, while cobalt nitrate was used to test the//

the effect of a common non-complex salt of the same metal.

(22) Oxidation of chlorophyll/carotenoid mixture in presence of cobalt nitrate.

Conditions:

0.3 gram thallous bromide.

3.35 ml. chlorophyll/carotenoid solution 1.126 g./litre in acetone.

0.2 ml. cobalt nitrate 0.14 g./50 ml. in water.

As had been expected, no dark reaction or catalysis of any kind was obtained in this case, the rates of oxidation on illumination being if anything, less than normal. It is therefore apparent that cobalt only initiates and catalyses a thermal oxidation of plant pigment if it is present in a complex form.

Experiments with oxycobaltiac ammonium nitrate.

It was found that the results obtained with cobalt naphthenate could be reproduced almost exactly with this salt. All the features of the reaction curve for the mixed pigments, including the oxygen uptake value reappeared. In this case also, a film of the salt itself on thallous bromide showed no evidence of pressure change in the dark or on illumination, whether in vacuo or in 100 m.m. of oxygen. Approximately the same weight of this salt as of cobalt naphthenate was used in most experiments. Again, the oxygen uptake was independent of the amount of salt used. As before, the salt had apparently no effect on pure chlorophyll. With carotenoids, a dark//

dark reaction was once more obtained, which gave in this case a molecular ratio of oxygen/carotenoid of 1.67:1. This reaction had a longer acceleration period than its counterparts with cobalt naphthenate, and consequently such an increased uptake might be expected.

Of the pigments present in the mixed film, the carotenoids certainly, from a consideration of their structure appear the most liable to undergo increased oxidation (98). If it is recalled that the oxygen uptake observed must be a minimum value, then the intermediate ratio of oxygen/carotenoid of 1.67:1 could be taken as indicating that each carotenoid molecule is now absorbing two molecules of oxygen instead of only one as previously observed.

Assuming this to be true for the moment, it is perhaps significant that if allowance is made for this 'double' oxidation of the carotenoids in the mixed films, the oxygen/chlorophyll molecular ratio remains unity.

It is not certain whether the points of oxidative attack on the pigment molecules are the same or even connected with those involved in the "normal" light - induced oxidations. However, it is of significance in this respect that the oxygen uptake can be increased by illumination. This may mean either (a) that the dark and light reactions can proceed simultaneously, implying that the oxidation centres are independent//

independent, or (b) that the products of the cobalt - initiated oxidation can undergo further oxidation on illumination.

The last salt, disalicylalethylenediimine cobalt, was examined in a similar fashion. Results with it were disappointing however, no dark uptake or catalysing action whatever being observed. Since the possibility existed of its depositing in an inactive form, depending to some extent on the solvent used (5), various solvents, including analar benzene and acetone were used in preparing the films. In one case, the solid was added to the vessel in the dry state and the chlorophyll deposited directly on it instead of on thallous bromide.

In no instance were the previous results repeated. The solid itself showed no uptake of oxygen under these conditions. This compared oddly with Diehl's results; however his work had been carried out at much higher pressures of oxygen.

The figures obtained with the two active cobalt salts are too consistent to be fortuitous. It was definitely established that in the presence of these salts, an oxidation proceeded in the dark, at rapid rates and to a greater extent than before. Nevertheless, in view of the difficulty already discussed, the reaction is not easily controlled, and consequently the method cannot yield an accurate figure, as in the case of the light-initiated reactions, for the total oxygen//

oxygen uptake. The investigation was therefore not pursued any further than this stage.

Reaction Mechanism.

The results obtained indicate that for the oxidation of carotenoids and of chlorophyll carotenoid mixtures, the two active cobalt salts not only act as catalysts, but also initiate the reaction.

Any reaction scheme proposed must take into account the following a dditional observations:-

(1) The presence of carotenoids is essential for any cobalt initiated thermal oxidation of chlorophyll. This means that carotenoids must be indispensable to the production of the radicals or intermediates involved in the reaction. Other instances are known of this pigment acting in a somewhat similar capacity (111).

(2) The reaction cannot be reversed by evacuation. The cobalt catalysed thermal oxidation of the pigments thus differs fundamentally from their photo-oxidation, which produced a dissociable peroxide. This may mean that this thermal oxidation takes place at entirely different centres which give rise to stable peroxides, or, since cobalt naphthenate in particular is a very active catalyst for the decomposition of peroxide products (110), it is also probable that the peroxide initially formed has decomposed to give non-dissociable products. If this is indeed the case, then the total pressure decrease cannot be referred//

referred to oxygen uptake alone in these catalysed experiments.

Certainly the general kinetics of this thermal oxidation appears, from the shape of the reaction curve, to resemble that of the heavy metal catalysed oxidation of tetralin, investigated by Robertson and Waters (110). In the present case however, the fact that the auto-oxidisable substance is present as a solid film greatly decreases the possibilities for chain reactions, because of the relative immobility of the molecules.

Of great interest is the mechanism by which this thermal reaction is initiated. A radical scheme, for the oxidation of the mixed pigments in the presence of cobalt naphthenate, on the lines of that proposed by George and Robertson (112) for tetralin, may be represented somewhat as follows:-

1. $M + O_2 \longrightarrow M \cdots\cdots O_2$
2. $MO_2 + R \longrightarrow RO_2^{\cdot} + M$
3. $RO_2^{\cdot} + 2R_1H \longrightarrow 2R_1^{\cdot} + RO_2H_2$
4. $R_1^{\cdot} + O_2 \longrightarrow R_1O_2^{\cdot}$
5. $R_1O_2^{\cdot} + R_1H \longrightarrow R_1O_2H + R_1^{\cdot}$ etc.

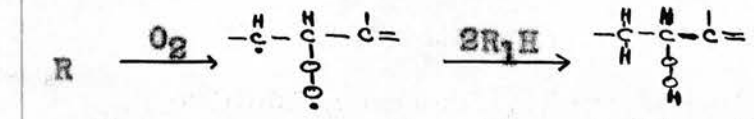
The chain terminating reactions, of a high efficiency, involve the destruction of $RO_2^{\cdot}$ and $R_1O_2^{\cdot}$.

Explanation of terms.

M represents the metal catalyst.

R " " carotenoid molecule, whose active region involves one, or two of the conjugated double bonds e.g :- $-\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-\underset{|}{C}-$

RO_2 represents the diradical obtained when oxygen adds on to a typical double bond, thus:-



i.e. RO_2H_2 (113)

R_1H represents the chlorophyll molecule etc..... Equation I represents the "activation" of oxygen by the complex cobalt salt. This could take place either by means of some addition reaction as indicated or more probably, by valency changes (112). Reaction III would explain why carotenoids are essential for the thermal oxidation of chlorophyll since they produce the reactive radical $R_1^{\cdot}$.

Of course, if the total pressure decrease obtained in these experiments does indeed mean an oxygen/carotenoid molecular ratio of 2:1, then the addition of any oxygen molecule to a carotenoid double bond must be imagined as occurring at two parts of the conjugated chain, and the proposed scheme suitably altered to interpret this.

A more simple representation can be obtained by adopting the valency change method used by Weiss (10) in his general oxidation schemes:-

$$Co^{++} \longrightarrow Co^{+++} + \epsilon$$

$$O_2 + \epsilon \longrightarrow O_2^-$$

$$Co^{+++} + car \longrightarrow Co^{++} + car^+$$

$$\begin{cases} Car^+ + O_2^- \longrightarrow Car\,O_2 \\ Car^+ \quad chl \longrightarrow car + chl^+ \end{cases}$$

$$chl^+ + O_2^- \longrightarrow chlO_2.$$

where//

where car represents the carotenoid molecule

chl ..... chlorophyll.

$CarO_2$)
) ..... their respective oxides.
$ChlO_2$)

Studies on the function of chlorophyll in photosynthesis.

The two fundamental processes of photosynthesis are the reduction of carbon dioxide and the oxidation of water. Most of the modern theories regard chlorophyll as being concerned solely with the latter reaction (114), and indeed there is a certain amount of evidence in support of this view (60).

However, the experiments of Willstatter and Stoll and of Rabinowitch demonstrated clearly the existence of a certain affinity of chlorophyll for carbon dioxide, although the nature of the attachment was still in doubt (115). There is the possibility that chlorophyll could absorb carbon dioxide chemically at the magnesium atom - by a process similar to carbamination. On the other hand, Rabinowitch stresses the point that this dark uptake of carbon dioxide by chlorophyll and its derivatives could also be explained by a physical adsorption.

(a) Carbon dioxide question.

In most of the previous experiments, as already mentioned, carbon dioxide gas was admitted at certain stages of the reaction, the partial pressure being of the order of 10-100 m.m. In no instance was any such//

such adsorption observed at these low pressures nor was any unusual effect recorded on illumination. In an attempt to approach natural conditions more closely, water vapour was invariably added with the carbon dioxide, as the two reactions of photosynthesis appear to be closely connected. In this case also, the rates of oxidation, apart from an initial increase due to evacuation, and the point of intersection of the curve with the ΣO_2 axis remained unchanged. Had an uptake of carbon dioxide or water vapour been involved, the curve would have shown a break, with an increased extrapolated value for the total pressure decrease. No photosynthesised material was ever detected on the film or in the resultant gases. The addition of carbon dioxide and water vapour was repeated when acid was present in the film (e.g. Experiments 4 - 8). As explained earlier, the acids concerned were chosen mainly for their connection with photosynthetic schemes, but the results with carbon dioxide were again negative. It had been hoped that some carboxylation of the chlorophyll peroxide might occur, thus:-

$$R.OOH + CO_2 = RO.CO.OOH$$

by analogy with the formation of percarbonates.

However these carbon dioxide experiments had all been carried out under acid conditions (ranging from the weak carbonic acid to phosphoric acid) and as a general rule, carboxylations proceed more readily in an//

an alkaline medium than under any other condition (116). It was therefore decided, as a suitable contrast to the acid experiments, to study the photo-oxidation of chlorophyll in the presence of some alkaline reagent, and in particular to study the effect of carbon dioxide on the reaction.

Since basic reagents such as carbonates, amines, etc., all absorb carbon dioxide on their own, it was decided to use ammonia vapour since it seemed the reagent least likely to cause complications.

(23) Oxidation of chlorophyll in presence of ammonia vapour.

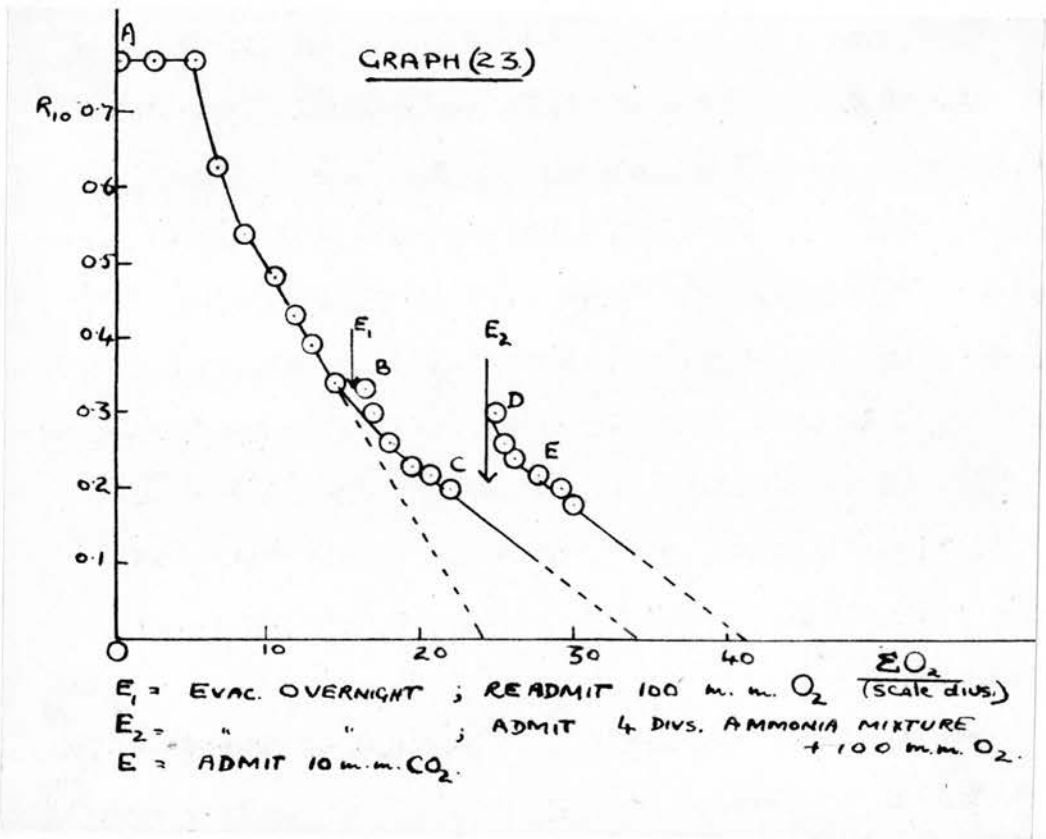
Conditions:

0.3 gram thallous bromide.

3.35 ml. chlorophyll/carotenoid solution 1.126 g/l. in acetone.

Volume of vessel + gauge = 86.9 ml.

The "ammonia" used was a mixture of ammonia and water vapour obtained from a concentrated ammonia solution. It was introduced into the system in the same manner as water vapour. Initially, 74.5 scale divisions of the ammonia mixture were admitted to the vessel and gauge. Approximately 5 divisions were adsorbed almost immediately, followed by a slow adsorption lasting some hours. After equilibrium was reached, the film was illuminated, but no pressure change was observed. At this pressure of mixed vapours, the additional adsorption on admitting another gas, such as oxygen, to the system is quite appreciable causing considerable//


GRAPH (23)
A
R_{10} 0·7
0·6
0·5
0·4
0·3
0·2
0·1
E_1
E_2
B
C
D
E
10
20
30
40
ΣO_2
(scale divs.)
E_1 = EVAC. OVERNIGHT ; READMIT 100 m.m. O_2
E_2 = " " ; ADMIT 4 DIVS. AMMONIA MIXTURE + 100 m.m. O_2.
E = ADMIT 10 m.m. CO_2.

considerable delay until equilibrium is again obtained. The apparatus was therefore evacuated and only 4 divisions of the mixed vapours admitted. When steady (i.e. after 0.8 divisions had adsorbed) 3.7 divisions of carbon dioxide gas were added, but no effect in the dark or on illumination could be recorded. When 100 m.m. of oxygen were added, a fairly fast pressure decrease occurred on illumination. The rates obtained were graphed to give the curve AB. The extrapolated oxygen uptake value for this section gave a molecular ratio of oxygen/pigment of less than unity. On evacuating the system and readmitting oxygen, no reversal was obtained, the rates apparently lying on a continuation of the original curve.

This "normal oxidation" in absence of ammonia was however reversible on evacuation at C. The points from D to E were obtained in the presence of 4 divisions of ammonia and 100 m.m. of oxygen. 10 m.m. of carbon dioxide were admitted at E without interrupting the illumination, but the subsequent rates were apparently unaffected by this gas.

Results:- //

Results.

Section AB		Section BC		Section DE	
ΣO_2	R_{10}	ΣO_2	R_{10}	ΣO_2	R_{10}
0.1	0.77	16.4	0.33	24.7	0.30
2.5	0.77	16.8	0.30	25.5	0.26
5.1	0.77	18.0	0.26	26.3	0.24
6.6	0.63	19.5	0.23	27.7	0.22
8.4	0.54	20.6	0.22	29.0	0.20
10.4	0.48	21.9	0.20	29.8	0.18
11.7	0.43				
12.9	0.39				
14.4	0.34				

At the end of the experiment, the gases in the reaction vessel were frozen out in a liquid oxygen trap and tested for peroxide and aldehyde reactions using the tests described previously. The film was extracted with water and tested similarly. All tests proved negative.

Conclusions.

Even in alkaline conditions, no definite uptake of carbon dioxide by chlorophyll could be detected. Certainly, the reaction of chlorophyll with oxygen in the presence of carbon dioxide and ammonia is not identical with the normal oxidation, but the differences are more probably due to the presence of the ammonia vapour, since no additional effect was obtained at the point E, when carbon dioxide was admitted to the system already containing the ammonia/water vapour mixture and oxygen.

The apparent decrease in oxygen uptake and the removal of the reversibility could be considered as due to two alternative causes:-

//

(a) the ammonia mixture which had adsorbed on the surface of the film having a purely physical covering effect and preventing the oxygen reaching some of the active centres of the chlorophyll molecules.

(b) the small amount of ammonia present had reacted to some extent with the keto group at C_9 to give a condensation product, the chlorophyll molecule being regarded in this connection as a substituted methyl aceto-acetic ester (117). This reaction would then restrict the oxygen uptake at position C_{10}. Furthermore, the stability of all molecules appears to be affected, since the reaction is not reversible in the presence of ammonia vapour.

Summary.

Under all the conditions so far investigated, no interaction between chlorophyll and carbon dioxide could be definitely established. In such systems however, chlorophyll was isolated from other participants, such as enzymes and acceptor molecules, in the photosynthetic scheme. It seems obvious that some or all of these naturally occurring reactants are essential for any chemical fixation of carbon dioxide.

(b) Water vapour Question.

It has already been mentioned that the modern views on photosynthesis regard chlorophyll as concerned primarily with those reactions which result in the liberation of oxygen from water vapour.

Experiments//

Experiments in which water vapour was illuminated in the presence of a film of chlorophyll ("copper-containing") on thallous bromide did not show any increase in pressure which could be taken as an indication of photo-decomposition. However, preliminary work on the illumination of water vapour in the presence of films of the same chlorophyll deposited on certain other solids, such as zinc oxide and ferric phosphate did show such an increase in pressure on illumination. Zinc Oxide in particular, has been reported (118) as being capable of sensitising the photo-decomposition of water in the liquid phase. It was therefore imperative that the effect of the solid itself should first be examined.

Films of zinc oxide were therefore prepared, by adding 0.3 grams dry solid to the reaction vessel, moistening sufficiently with water to form an even surface, and then drying and evacuating in the usual way. The pressure of water vapour added was varied from 2 to 6 m.m. of mercury. When the system had reached a steady state, after the initial adsorption of vapour, the Osira light was switched on and pointer readings were taken for several hours. It was found in all cases, that after an induction period of 20 to 40 minutes, a slow steady increase in pressure occurred until a certain maximum value was obtained, after which continued illumination produced no further pressure change. The total increase in pressure//

pressure was very small but fairly consistent, and at these low pressures employed, it could not be accounted for by slight temperature fluctuations in the thermostatic control. After standing overnight in the dark, the introduction of a further few millimetres of water vapour followed by illumination when steady was found to give a repetition of the effect.

Other solids, including ferric phosphate and ferric oxide were also used, and were found to give essentially similar pressure effects to those obtained with zinc oxide, although with ferric oxide the induction period was eliminated.

The experiments were repeated with approximately 3 milligrams (a) of bonemeal-purified chlorophyll and (b) of the chlorophyll/carotenoid mixture, both being "copper-chlorophyll" preparations deposited on the solid concerned.

With zinc oxide and ferric oxide the results were exactly the same as for the solids alone, but with the chlorophyll/carotenoid mixture on ferric phosphate, the long induction period was eliminated and an immediate, although small, pressure increase was obtained on illumination. This was followed again by the "steady state" period, but on discontinuing the illumination, a pressure decrease equal in extent to the initial increase was observed.

With the zinc oxide and ferric phosphate films, oxygen was subsequently admitted to the system and the //

the pigment allowed to photo-oxidise to a slight extent before evacuating and readmitting water vapour. The presence of any oxide however was not found to have any observable effect on the pressure changes normally produced.

In no instance did the results show any direct relationship between the amount of water vapour present and the extent of the pressure increase, nor could any hydrogen peroxide be detected on freezing out the final products in the usual way. This was not unexpected, however, since, from the pressure change obtained, the amount of peroxide produced, if any, must be exceptionally small.

Rabinowitch suggested (119) that the primary process in the photo-decomposition of water vapour sensitised by zinc oxide, is the production of OH and H radicals, made possible by their large heats of adsorption on the solid, thus:-

$$\underset{\text{(adsorbed)}}{H_2O} \xrightleftharpoons[\text{dark}]{\text{light}} \underset{\text{(adsorbed)}}{H} + \underset{\text{(adsorbed)}}{OH}$$

The subsequent formation of hydrogen molecules or of oxygen from the decomposition of the OH radicals, would explain the increase in pressure obtained, but it is impossible to formulate as yet any schemes on the results of this preliminary work, especially as recent work (120) has indicated that the pressure effects obtained are dependent to some extent on the pre-treatment of the zinc oxide.

Furthermore//

Furthermore, since the effective wavelength for zinc oxide is, according to Goodeve (39), 3650Å, it would be advisable in any future extension of this work to use a stronger source of this wavelength, and so increase the small effect obtained.

The results with ferric phosphate and ferric oxide can no doubt be explained by an ionic mechanism analogons to that proposed by Weiss to explain the photoreactions of solutions containing positive ions of variable valency (121).

However, from the aspect of a chlorophyll sensitised photo-decomposition of water, this method of approach does not appear capable of yielding promising results, since the effects were practically the same whether or not chlorophyll was present on the solid.

Two additonal factors must be taken into consideration before dismissing the subject however:-

(1) that the chlorophyll used was the copper-stabilised form.

(2) that the amount of pigment used was possibly in excess of that required of a sensitiser. Consequently, the pigment layer exerted a "covering effect", which masked any sensitising action.

With regard to the first point, it was observed that when water vapour was illuminated in presence of a film of "spinach chlorophyll" adsorbed on thallous bromide, a distinct increase in pressure, of similar extent//

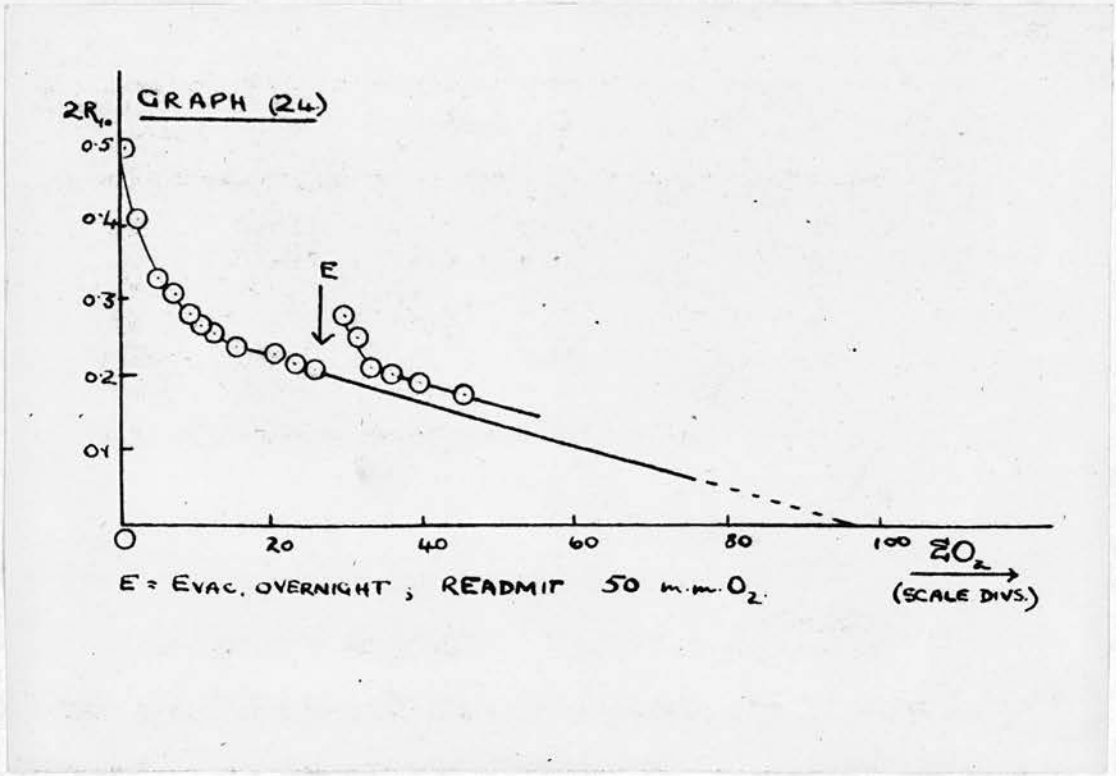
extent, to a steady maximum value again occurred. Since this did not take place on illuminating water vapour in the presence of thallous bromide alone, some photo-decomposition of water vapour may indeed be produced even under these in vitro conditions, if copper-free chlorophyll is used as sensitiser.

From the photosynthetic viewpoint, this was the most promising result obtained, and is certainly worthy of future attention.

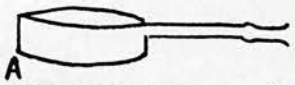
The Function of thallous bromide.

As already mentioned, most of these experiments were carried out on thallous bromide. Tests for free bromine (128) on the gases frozen out in a liquid oxygen trap, (a) at the end of a typical oxidation of a pigment film, and (b) after illuminating thallous bromide itself, in vacuo and in the presence of oxygen, all proved to be negative. In case (b) no pressure changes were obtained in the dark or on illumination, which would indicate bromine liberation or oxygen absorption.

However, to confirm that thallous bromide was indeed acting only as a sensitiser, the oxidation of the first chlorophyll preparation was examined on talc and on powdered glass. In the experiment with the chlorophyll deposited on talc in the normal sized reaction vessel, very slow rates (R_{10} = 0.07) were obtained. To produce reasonable reaction rates with these "inert" substances, a new reaction vessel was constructed"//


GRAPH (24)
$2R_\infty$
0·5
0·4
0·3
0·2
0·1
E
0
20
40
60
80
100
$2O_2$
(SCALE DIVS.)
E = EVAC. OVERNIGHT ; READMIT 50 m.m. O_2.

constructed, of approximately one third the size of the original. To facilitate the preparation of films, it was almost cylindrical in shape, with convex faces, the film being deposited on face A:-



To decrease fluctuations due to variation with such a small volume, the experiment was carried out at 50 m.m. of oxygen.

Conditions:

0.3 grams powdered JENA glass.

1.425 ml. bonemeal-purified chlorophyll solution 3.16 grams/litre in acetone.

Volume of vessel + gauge = 30.14 ml.

Results:- (24)

ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$	ΣO_2	$2R_{10}$
0.5	0.50	12.0	0.26	29.4	0.28
2.3	0.41	14.0	0.25	31.0	0.25
5.0	0.33	15.5	0.24	33.0	0.21
7.3	0.31	20.4	0.23	35.2	0.20
9.1	0.28	23.0	0.22	39.0	0.19
10.5	0.27	25.8	0.21	45.0	0.17

Total ΣO_2 = 97.0 scale divisions.

$\therefore \frac{\text{Oxygen}}{\text{chlorophyll}}$ = 0.98 molecularly.

The essential features of the oxidation on thallous bromide, namely the molecular ratio and the reversibility, were therefore confirmed on powdered glass.

General//

GENERAL DISCUSSION AND CONCLUSIONS.

General Discussion and Conclusions.

Apart from the work of Lonie and Ritchie, to which reference has already been made (82), it may be claimed that the present examination of chlorophyll and its related compounds involves conditions and experimental methods which have not been applied previously to this general problem. It was therefore necessary to spend some considerable time firstly in the establishment of such methods, and secondly in ascertaining the general trend of all the factors involved, by comparing the results of three pigment preparations under carefully selected conditions. Without such an initial survey, any detailed investigation could quite possibly have given rise to misleading conclusions, requiring later amendment.

On the basis of the experimental results obtained, a comprehensive scheme applicable to such pigment oxidation may now be reasonably proposed.

It must first be emphasised that the method used throughout these experiments was based on the measurement of pressure change. The decrease in pressure actually obtained under the simplest conditions, i.e. with oxygen alone as the reacting gas, may in the most general sense, be due to absorption of oxygen with the subsequent liberation of another resultant gas. However no gaseous products were ever detected by the "freezing-out method", nor was any increase in pressure observed under any conditions//

conditions, which could be related to gas production. Furthermore, the "molecular ratios" of "oxygen pressure decrease" to amount of pigment present were always integral numbers, capable of reasonable interpretation.

The second point which must be considered is that, because of the exceptionally slow rates of pressure decrease on inert substances, such as glass or talc, the majority of the results have been obtained using the accelerating substance, thallous bromide. The work of Ritchie and Thom (37) showed that in the presence of gelatin, thallous bromide is decomposed on illumination and a latent image of aggregated thallium atoms is formed. The gelatin was regarded as acting as a bromine atom acceptor, and the possibility existed that chlorophyll and carotenoids could act in a similar capacity, by virtue of their unsaturated nature. Such a thallium latent image, if formed, would be expected to react with water vapour, and carbon dioxide, especially if oxygen were present, to give an (additional) decrease in pressure, according to the following mechanisms:-

(a) $2Tl + 2H_2O = 2TlOH + H_2$

$2Tl + H_2O + CO_2 = Tl_2CO_3 + H_2$

(b) In presence of oxygen:-

$4Tl + O_2 + 2H_2O = 4TlOH$

$4TlOH + 2CO_2 = 2Tl_2CO_3 + 2H_2O$

However//

However, as already mentioned, water vapour and/or carbon dioxide gas were entirely without effect on the reaction rate or on the final gas uptake of any pigment film.

Thallous bromide when illuminated by itself or with either of the pigments examined, gave no increase in pressure in vacuo, nor could any free bromine be detected in the gases frozen out at the end of a typical oxidation. Finally, no change in the film of pigment on thallous bromide was visible after any illumination in the absence of oxygen.

To these observations must be added the fact that the major features of the results with chlorophyll/ thallous bromide films - for example, the unit molecular ratio and the reversibility - are essentially the same on "inert" materials, the only difference being the rate of reaction with oxygen.

All these considerations indicate that, under the present conditions, thallous bromide acts as a true sensitiser, being in the same state at the end as at the beginning of the oxidation.

Since thallous bromide resembles silver bromide in possessing a conductivity energy level in which electrons are mobile, the initial effect of absorbed light is almost certainly the expulsion of an electron from the bromine ion into this level:-

$$Br^- + h\nu \longrightarrow Br + \epsilon_1 \quad \ldots\ldots\ldots (1)$$

When//

When thallous bromide is illuminated by itself, in the presence of oxygen, water vapour, or carbon dioxide, this electron can only return to the bromine atom, producing no final change. But, when pigment molecules are also present, since the latter may themselves part with electrons (122), possibly only after illumination, we have the following explanation of the accelerating effect of thallous bromide:-

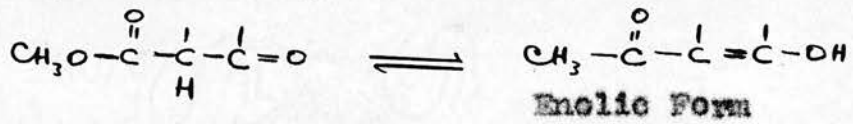
$$A \longrightarrow A^{+} + \epsilon_{1} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$
$$Br + \epsilon_{2} \longrightarrow Br^{-}$$
$$O_2 + \epsilon_{1} + A^{+} \longrightarrow \left[A^{+}\, O_2^{-}\right]$$

where, using the notation of Weiss (10), $\left[A^{+}\, O_2^{-}\right]$ represents the oxide product and A the molecule of pigment. If reaction (2) does require light absorption, A^{+} is probably a tautomeric triplet state (75) in which the repulsive effect of the unpaired electrons causes the actual loss of an electron to the conducting lattice. In the absence of such a lattice, the ground state will be rapidly re-attained, with a marked decrease in the rate of reaction - as observed in the experiments with powdered glass.

The general chemistry of chlorophyll derivatives (13) indicates the existence of three predominately reactive hydrogen atoms in the molecule. One of these is attached to the carbon atom C_{10}, and the two others belong to the imino groups formed on the removal of the central magnesium atom. It is satisfactory that the present results agree with these findings - the molecular ratio of oxygen to pigment being//

being increased from unity for chlorophyll itself, to three, on its conversion by acids to the magnesium-free pheophytin. It may be mentioned in this connection that carbonic acid was never able to bring about such conversion, acids of greater strength such as phosphoric, pyruvic and ascorbic acids being required.

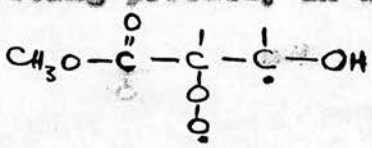
The "normal" oxidation presumably occurring at C_{10}, has been confirmed as being largely reversible. Again, this can be explained by the general properties of this position in the molecule, which is capable of ready enolisation, as follows:-



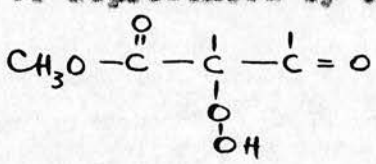
Enolic Form

(For convenience, only the relevant portion of the molecule has been drawn).

If the above reaction is induced by light, oxygen could then be absorbed at the double bond of the enolic product, the latter being probably in a diradical form, to give



which is a possible configuration for the reversible oxide obtained in these experiments. The irreversible form finally produced can be represented by the hydroperoxide:-



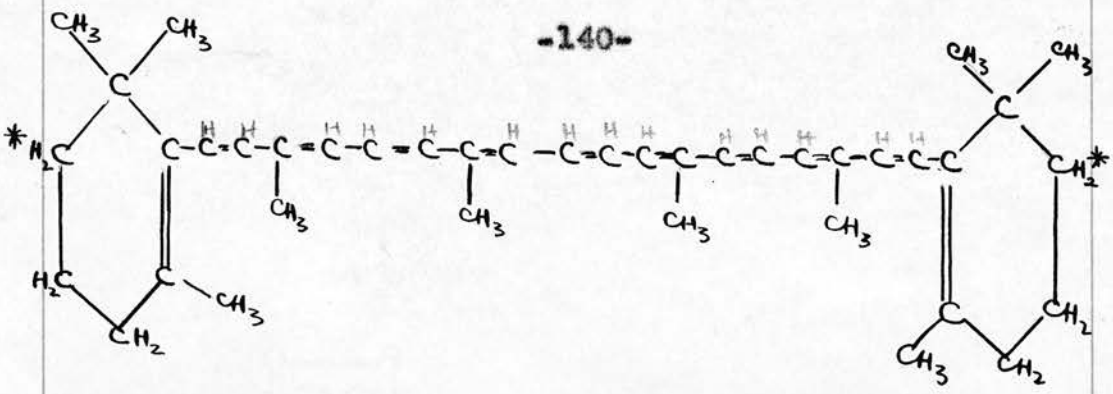
where the hydrogen atom has returned from the enolic position. The//

The possible resonance association of the hydrogen atom with either of the adjacent carbonyl groups would explain the stability of such a compound.

Since it forms initially a reversible peroxide, chlorophyll belongs to the class of organic compounds typified by rubrene (8), and is thus distinct from the larger group of auto-oxidisable substances which yield irreversible products.

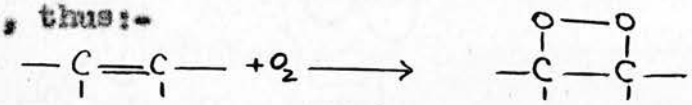
By contrast, the oxidation of the imino-positions of pheophytin shows little evidence of reversibility. Such a difference is not unexpected, since a carbon atom is not directly concerned in the latter case. The shape of the oxidation rate curve for pheophytin is such as to suggest two oxidations which proceed more or less independently. It is difficult to predict which oxidation takes place preferentially, but the shape of the curves indicates that it is the C_{10} oxidation which occurs first, followed by the slower reaction at the two equivalent imino positions. As already remarked, the resonance of the porphyrin ring system is affected considerably by the removal of the magnesium.

It was rather surprising that, with so many centres of unsaturation, the carotenoid fraction under the present conditions, absorbed only one molecule of oxygen per molecule of pigment. The formula for β-carotene is as follows, where asterisks denote the positions of the hydroxyl groups in xanthophyll:-

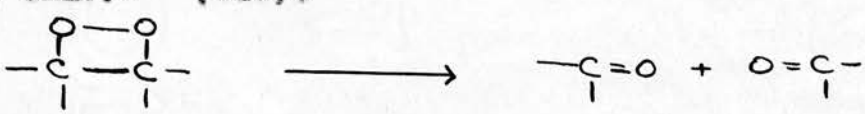


β-carotene (123).

Accordingly to Rabinowitch (98) the oxidation of carotene occurs with the formation of double bond peroxides, thus:-



It is obvious that only one of these bonds can be involved in the present oxidation. The product obtained was found to be reversible but to a much smaller extent than the chlorophyll peroxide.
It is possible that the formation of carotene peroxide is followed ultimately by the irreversible scission of the chain with the production of 2 ketones, thus:- (113).



<u>The kinetics of such photo-oxidations.</u>

An attempt will now be made to summarise the experimental results and their interpretation discussed in the preceeding pages, by developing a general reaction mechanism. As it is the simplest case, the "normal" photo-oxidation of chlorophyll itself on thallous bromide will be taken as a basis for discussion, but with slight modification the scheme//

scheme is also applicable to the other pigments investigated.

The following sequence of equations can be regarded as representing the "photo-oxidation" of chlorophyll as observed under the present conditions:-

$$chl + h\nu \longrightarrow chl^{*} \quad \ldots\ldots\ldots\ldots\ldots (1)$$
$$chl^{*} \longrightarrow chl \quad \ldots\ldots\ldots\ldots\ldots (2)$$
$$chl^{*} \longrightarrow tchl \quad \ldots\ldots\ldots\ldots\ldots (3)$$
$$tchl \longrightarrow chl \quad \ldots\ldots\ldots\ldots\ldots (4)$$
$$tchl + O_2 \longrightarrow tchlO_2 \quad \ldots\ldots\ldots\ldots\ldots (5)$$
$$tchlO_2 \longrightarrow tchl + O_2 \quad \ldots\ldots\ldots\ldots\ldots (6)$$
$$tchlO_2 \longrightarrow chlO_2 \quad \ldots\ldots\ldots\ldots\ldots (7)$$

The significance of these equations and of the terms involved will now be explained in turn.

The reaction is initiated by a ground state chlorophyll molecule (represented by chl) absorbing a quantum of light energy (1) to form an excited molecule (chl^{*}) of higher electronic state. Since the observed quantum efficiency was low (82), little of this absorbed energy effects any chemical reaction. Consequently the majority of these excited molecules must revert to the ground state by some internal degradation process - such as reaction (2), while a few only enter the rela tively long-lived 'reactive' state, by process (3). This long-lived species, which still possesses an energy higher than the ground state, is denoted by the term "tautomeric" chlorophyll - or "tchl" and a considerable amount of evidence for its existence has appeared in the literature (75).

This tautomeric form may also of course, revert to the//

the ground state ④, but its relatively long life enables it to react with molecular oxygen ⑤, forming a chlorophyll peroxide, $tchlO_2$. This reaction is initially reversible ⑥, but an irreversible peroxide ($chlO_2$) is gradually formed as the result of a final and possibly photochemical process ⑦.

The reversible auto-oxidation, i.e. reactions ⑤ and ⑥, requires more detailed consideration. With a gas in contact with two distinct solid phases, thus:-

$$tchl_{solid} + O_{2\,gas} \rightleftharpoons tchlO_{2\,solid} -$$

this system is directly analogous to the well-known equilibrium involved in the dissociation of calcium carbonate:-

$$CaO_{solid} + CO_{2\,gas} \rightleftharpoons CaCO_{3\,solid}$$

Since both systems possess two components and three phases, according to the phase rule they are univariant. In other words, at a given temperature, equilibrium is attained at a definite gas pressure, which is the dissociation or equilibrium pressure for that particular temperature, and is independent of the absolute amounts of the reactants present. For instance, on decreasing the carbon dioxide pressure at a constant temperature, carbonate dissociates until either this equilibrium pressure is re-established or the carbonate phase has disappeared entirely from the system. Conversely, increasing the carbon dioxide pressure at a fixed temperature causes the formation of//

of carbonate at the expense of calcium oxide. By applying similar arguments to the less familiar chlorophyll peroxide system, the explanations for several experimental observations become more obvious.

Firstly, the oxygen pressures normally used were in excess of any dissociation pressure of the reversible peroxide, since this phase was quite stable under the prevailing conditions. The experimentally measured rate of oxidation is therefore proportional to some function of the number of reactive chlorophyll molecules temporarily in existence and of the oxygen pressure in excess of the equilibrium pressure.

In the dark, after a period of illumination and still at this high oxygen pressure, no appreciable dissociation of the peroxide can occur. Furthermore, under these conditions, only an exceedingly small amount of reactive chlorophyll will exist, and reversion to the ground state ④ and continued oxidation ⑤ will therefore take place at negligible rates. This explains why the system is found to be apparently unchanged on resuming illumination.

By contrast, a dark period during which the oxygen pressure is reduced below the value of the equilibrium pressure, leads to the dissociation of the reversible peroxide. The reactive chlorophyll so formed reverts, irreversibly in the absence of light, to the ground state. In this form it is unable to react with oxygen when re-introduced at the higher pressures//

pressures, and consequently no dark uptake is observed. On continuing the illumination however, the rates of oxidation and the value for the total gas uptake should indicate that some reversal has occurred. This was indeed always observed after a dark interval in vacuo, and in general, Lonie and Ritchie (82) also showed that the reversibility depended on the pressure reduction (for constant time periods), becoming inappreciable above approximately 125 m.m. If the rate constants for the equations proposed are represented respectively for reactions ① to ⑦ by I_{abs}, k_2, k_3 ..... and k_7 , where I_{abs} represents the intensity of the absorbed light, then a kinetic expression for the overall reaction rate may be derived as follows:-

In the steady state, these relationships must hold:-

$$\frac{d[chl^*]}{dt} = I_{abs} - k_2[chl^*] - k_3[chl^*] = 0$$

$$\text{whence } [chl^*] = \frac{I_{abs}}{k_2 + k_3}$$

$$\frac{d[tchlO_2]}{dt} = k_5[tchl][O_2] - k_6[tchlO_2] - k_7[tchlO_2] = 0$$

$$\text{whence } [tchlO_2] = \frac{k_5[tchl][O_2]}{k_6 + k_7}$$

$$\frac{d[tchl]}{dt} = k_3[chl^*] - k_4[tchl] - k_5[tchl][O_2] + k_6[tchlO_2]$$

$$= \left(\frac{k_3}{k_2 + k_3}\right) I_{abs} - k_4[tchl] - k_5[tchl][O_2] + \frac{k_5 k_6}{k_6 + k_7}[tchl][O_2] = 0$$

$$\text{whence } [tchl] = \frac{\left(\frac{k_3}{k_2 + k_3}\right) I_{abs}}{\left\{k_4 + k_5[O_2] - \left(\frac{k_5 k_6}{k_6 + k_7}\right)[O_2]\right\}}$$

Now when//

Now when $[O_2] = [O_2]_{equilibrium} = [O_2]_e$

$$k_5 [tchl] [O_2]_e = k_6 [tchlO_2]$$

In the general case,

$$\frac{-d[O_2]}{dt} = k_5 [tchl] [O_2] - k_6 [tchlO_2]$$

$$= k_5 [tchl] [O_2] - k_5 [tchl] [O_2]_e$$

$$= k_5 [tchl] \{[O_2] - [O_2]_e\}$$

Hence $$- \frac{d[O_2]}{dt} = \frac{k_5 \left(\frac{k_3}{k_2 + k_3}\right) I_{abs} \{[O_2] - [O_2]_e\}}{\left\{k_4 + k_5 [O_2] - \frac{k_5 k_6}{k_6 + k_7} [O_2]\right\}}$$

$$= \frac{\left(1 + \frac{k_6}{k_7}\right)\left(\frac{k_3}{k_2 + k_3}\right) I_{abs} \{[O_2] - [O_2]_e\}}{\frac{k_4}{k_5}\left(1 + \frac{k_6}{k_7}\right) + [O_2]}$$

which may be reduced to the form

$$\frac{-d[O_2]}{dt} = \frac{k \; I_{abs} \{[O_2] - [O_2]_e\}}{k' + [O_2]}$$

where k and k' involve only constants.

Such an expression requires that apart from the conversion of unstable to stable oxide, the rate of oxidation should become zero at a finite pressure of oxygen equal to the dissociation pressure of the unstable oxide. Such an effect has recently been confirmed for pheophytin by Miss C.M. Laurie in this Department (unpublished results). The limiting pressure below which practically no oxidation occurs is less than 0.1 m.m. of mercury. (With the copper chlorophyll, the rates at such low pressures were so slow//

slow that it was impossible to estimate its dissociation pressure.

The expression has also been substantiated by the work of Lonie and Ritchie (82) who showed that at oxygen pressures above 10 m.m. of mercury, the copper-containing chlorophyll oxidised on thallous bromide at a rate proportional to $\frac{[O_2]}{a+[O_2]}$ and that a was approximately 140 for pressures in millimetres, $[O_2]_e$ being negligible in comparison with these high oxygen pressures.

Furthermore, the present work has confirmed that the rate of oxidation is directly proportional to the intensity of the illumination.

However, because of the conditions obtaining in these experiments, it is doubtful whether the variation of rate with oxygen pressure is determined strictly by the rate constants of the above reaction mechanism. The oxidation is a photosensitised one, with possible loss of light energy in the thallous bromide itself. Further, the rate may be determined by such factors as the diffusion of oxygen through the adsorbed film to the photochemically active centres of oxidation. For a multi-molecular layer, in such a sensitised system, any reaction must be visualised as occurring at the immediate point of contact of the innermost layer with the sensitiser, unless of course, the photoactive centres can be transferred//

transferred throughout the entire film by an electron conduction process. There is no further definite information on this subject, but Lenie and Ritchie (82) found that the absolute quantum efficiency, for light of wavelength 4360Å, of copper-chlorophyll on the plane glass surface of a reaction vessel increased with decreasing film thickness. It is obvious from this result that such a complete transference of reactivity does not occur and therefore the higher rates must be due to increased diffusion of oxygen through the film to the reactive centres. The "diffusion gradient" of oxygen will be determined not only by the oxygen pressure, but also by the actual film thickness; the effect will possibly be complicated still further by differences in the diffusion characteristics of the reactant chlorophyll and the oxide product.

Because of such a diffusion process, the experimentally recorded rates of pressure decrease may not give an immediate measure of the actual rate of oxidation. Following the general experimental procedure, it was necessary to allow the prepared film to remain in contact with oxygen in the dark, prior to illumination, until the system reached a steady temperature. Under these conditions, the concentration of oxygen in the film can be regarded as approaching a uniform value. On illumination, rapid oxidation may take place at the thallous bromide surface, without any pressure//

pressure decrease becoming apparent, until the establishment of an appreciable oxygen concentration gradient results in oxygen diffusing through the film. The "acceleration period" always found with the amounts of chlorophyll (first preparation) initially employed can be accounted for, by such mechanism. By contrast, the experiment on powdered glass as adsorbent showed no such initial slow period. Here no sensitiser is present, the reaction being initiated by the light absorbed by the chlorophyll itself - and consequently oxidation occurs first at the outer light-exposed surface of the film, showing an immediate pressure decrease. With the films of the third chlorophyll preparation on thallous bromide, such a short initial period of relatively faster rates was also observed. It is possible that in this instance, the light absorbed by this "unstabilised" chlorophyll is relatively more effective.

Alternatively of course, the rate of diffusion of oxygen may depend on several factors other than the pressure and the film thickness - for example, the "porosity" of the film - which is determined by the number of channels giving free access to the inner layers. This may itself explain the variations observed in the initial period with the different preparations. The small decrease in pressure obtained by Lonie and Ritchie in the dark period immediately following an illumination could be explained//

explained by the diffusion process or by the long-life of the tautomeric form of chlorophyll. Similarly, the acceleration period could be due to a necessary initial accumulation of such tautomers. However, the periods involved suggest a lifetime much greater than would normally be expected. There is one further point connected with the kinetics to which reference must be made. In the pheophytin experiments, although the oxide formed by this pigment was not appreciably reversible, on evacuation overnight followed by standing in oxygen for the usual time prior to illumination, a marked increase in the rates of photo-oxidation was observed over a short period (see graphs 4,5,8 etc.). This temporary increase was independent of the extent of oxidation and therefore of the amount of oxide formed. Furthermore, it cannot be explained by the oxygen diffusion process discussed above, as this would indicate an acceleration period on initial illumination - as actually observed in some cases when evacuation was not applied.

The only feasible explanation for this effect is that the removal of oxygen from the film during evacuation "opens up" the film, so that on re-illumination oxygen can reach the reactive centres immediately, without any diffusion being involved. This explanation implies that the accumulating product tends subsequently to block such avenues of oxygen admission.

The//

The above considerations have been put forward assuming that the oxidisable material existed in a multimolecular layer. An estimate of the molecular thickness of one of these films was obtained by calculating the area covered by the pigment, if spread as a unimolecular layer, and dividing this value by the total surface area of the actual thallous bromide grains, as follows:-

Using a high power microscope with a calibrated eye-piece scale, the crystals were observed to be cubic, of approximately the same size, with an average cube side of 3.5×10^{-4} cm. The surface area of one cube is therefore $12.2 \times 10^{-8} \times 6$ square centimetres $= 7.3 \times 10^{-7}$ sq. cm. From the density of thallous bromide (124) given as 7.55 grams/c.c., the total volume of 0.3 grams used in any one experiment

$$= \frac{0.3}{7.55} = 0.0397 \text{ c.c.}$$

Since the volume of each cube is $(3.5 \times 10^{-4})^3$ c.c the number of cubes involved is

$$\frac{0.0397}{42.88 \times 10^{-12}} = 0.927 \times 10^{9}$$

Therefore, the total surface area $= 7.3 \times 10^{-7} \times 0.927 \times 10^{9}$

$$= 0.677 \times 10^{3} \text{ sq. cm.}$$

To this surface, was added 3.35 ml. of a solution containing approximately 1 gram of chlorophyll per litre - i.e. 3.35×10^{-3} grams chlorophyll

$$\text{i.e. } \frac{3.35 \times 10^{-3} \times 6.06 \times 10^{23}}{900} = 2.3 \times 10^{18} \text{ chlorophyll molecules.}$$

The//

The area occupied by a chlorophyll molecule on a surface is uncertain, because of the variety of orientations possible on adsorption, but the "frontal area" of ethyl chlorophyllide (125) has been estimated as approximately 240×10^{-16} sq. cm. The area covered by a unimolecular film of 2.3×10^{18} molecules all lying flat would therefore be $2.4 \times 10^{-14} \times 2.3 \times 10^{18}$

$= 5.5 \times 10^{4}$ sq. cm.

Since the area of the crystals was 0.677×10^{3} square cms., the average molecular thickness is -

$$\frac{5.5 \times 10^{4}}{0.677 \times 10^{3}} = 81 \text{ molecules.}$$

Other results indicate however (126) that chlorophyll and its derivatives tend to form films with their molecules orientated "on end" and inclined at 55° to the basal plane. The minimum surface requirement for a chlorophyll molecule in such circumstances was found by Hanson to be 106×10^{-16} sq. cm.

Taking this figure, the molecular thickness would be in the present case approximately 36 molecules, and from all aspects this appears to be the most probable figure.

Such calculations give a maximum molecular thickness, because of course the effective adsorptive surface of the thallous bromide crystals must be greater than the "geometric plane" - area as estimated above. The experiments on the variation of the amount of oxidisable pigment for a given weight of thallous bromide//

bromide are therefore of importance in this connection (p.63).

It has been shown that for chlorophyll-carotenoid mixtures, the existence of the constant rate portion is determined by the amount of carotenoids present - in particular, according to the results of the acid experiments, by the amount of carotene itself. With very small amounts of carotenoid, no definite constant rate was obtained.

During such a period, the rate of supply of light energy must be constant. If, as in the case of the very thin films of carotene, such a state is not attained, then a layer of pigment cannot be uniformly adsorbed over all the crystal surfaces, resulting in a loss of light energy. It may therefore be suggested that the amount of carotenoid at which such a constant rate begins to appear indicates the existence of a unimolecular layer on the surface although the term "unimolecular" as used here refers strictly to the active sites on the crystals. It is impossible to decide at present whether every part of the thallous bromide surface can "transfer" light energy, or whether such a property is restricted to certain reactive points, such as lattice irregularities.

Applying the same method of calculation to carotenoid films, an estimate of the "normal" molecular thickness i.e. with reference to the whole surface, gave a value of approximately 3 to 9 molecules (depending//

(depending on orientation) for a weight of carotenoid just below the limit necessary for the production of a definite constant rate period. The surface requirement of a carotenoid molecule was calculated from the data published by Taylor (127). This value for the thinnest carotenoid films is several times smaller than that calculated for an average chlorophyll film, yet no constant rate was ever observed with the latter. It would appear, therefore, that the constant rate period is peculiar to carotene films. A possible explanation for this lies in the relative molecular sizes of the two pigments. The small size of the carotene molecule would allow good contact between the reactive sites on the thallous bromide surface and the oxidation centres in the molecule, preventing loss of activating energy. The greater area and variety of possible orientations of the chlorophyll molecule may, by contrast, prevent this essential association. Consequently no constant rate period was ever apparent with films of the latter pigment alone, irrespective of their molecular thickness.

Returning to the question of the carotenoid films, it was found that provided sufficient was used, the extent of the constant rate period was directly proportional to the actual weight of carotenoids present in the film. This result indicates that the constant supply of energy is maintained in the thicker films, the rates only decreasing eventually when "bare patches", causing//

causing loss of light energy, appear.

In conclusion it must be emphasised again that in this method of investigating the photochemistry of naturally-occurring pigments, the pigment in question was without exception adsorbed on a solid surface. These conditions are at first sight very different from those obtaining in vivo. Yet a certain relationship does exist, for chlorophyll itself in the plant is also adsorbed, probably in a semi-solid state, on some protein carrier.

Recent spectrographic work (129) in this connection has shown that the absorption curve of naturally occurring chlorophyll resembles more closely that of chlorophyll deposited on talc or other adsorbents, than that of dissolved chlorophyll. Possibly this gives a further justification for studying the photo-oxidation of such pigments in the solid phase.

Summary of experimental findings.

(1) A method has been developed for studying the photo-oxidation of plant pigments on thallous bromide; it depends essentially on the accurate measurement of the pressure decreases involved, using a sensitive Bourdon glass-spring gauge.

(2) The results show that, under these conditions, both freshly prepared and copper-stabilised chlorophyll absorb one molecule of oxygen per molecule of pigment. This is also true for the carotenoid fraction. For acid-treated chlorophyll, or pheophytin, this ratio is increased threefold. These oxidations were shown to be partly reversible.

(3) The thallous bromide has been shown to act merely as a photo-sensitiser.

(4) A thermal oxidation of carotenoids and of carotenoid/chlorophyll mixtures was observed in the presence of certain cobalt salts.

(5) It has proved impossible as yet, with this in vitro system, to simulate any photosynthetic reaction, although the effect of carbon dioxide and water vapour was examined for the majority of the experiments.

(6) A general scheme has been proposed to account for the main features of the photo-oxidation.

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